

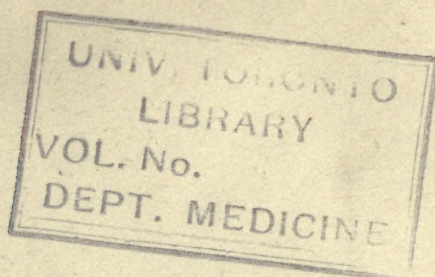








SCIENCE AND MEDICINE DIVISION















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THE BRITISH JOURNAL  
OF  
EXPERIMENTAL  
PATHOLOGY







THE BRITISH JOURNAL  
OF  
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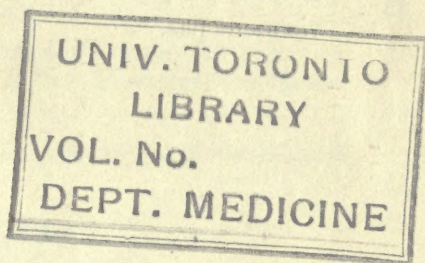
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“ Ah ! si je savais dire comme je sais penser ! Mais il était écrit là-haut que j'aurais les choses dans ma tête, et que les mots ne me viennent pas.”

Diderot. *Jacques le Fataliste et son Maître.*



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# THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY

## ON THE EFFECTS OF CHANGES IN INTRAVENTRICULAR PRESSURE AND FILLING ON THE VENTRICULAR RHYTHM IN PARTIAL AND COMPLETE HEART BLOCK.

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RECENTLY one of us has had the opportunity of observing a number of clinical cases suffering from auriculo-ventricular block, and our attention was drawn in particular to the change in ventricular frequency which occurred with or without the onset of syncope.

With the aid of modern instruments the mechanism of any given change of rhythm may be determined in cases of both partial and complete auriculo-ventricular block; on the other hand, the actual causes of the alterations in rhythm are nearly always obscure.

In two cases suffering from complete auriculo-ventricular block (confirmed histologically), the idio-ventricular rhythm was interrupted by groups of rapid ventricular systoles, periods of asystole intervening between each group. In one case the ventricular frequency would sometimes be as high as 130 per minute at the commencement of the group and gradually slow down to about 70 per minute before stopping altogether. The ventricular pauses lasted from a few seconds to three minutes. The ventricular beats were counted with the stethoscope over the præcordium, and we presume the rhythm was due to ectopic beats arising in the ventricle in the same manner as reported by Cohn and Lewis (1912-13).

The effect upon idio-ventricular rhythm of rapid artificial stimuli applied to the ventricle has been studied experimentally by Erlanger and Hirschfelder (1905-6). They found that cessation of a new and more rapid artificial rhythm was followed by a ventricular pause, the idio-ventricular rhythm taking time to develop again. In the report of Cohn and Lewis the idio-ventricular rhythm was interrupted by a number of premature beats arising in the ventricle; at the end of the ectopic rhythm there was a ventricular pause



before the idio-ventricular rhythm developed; they pointed out the resemblance between their case and the condition studied experimentally by Erlanger and Hirschfelder. Cushny (1911-12), in his researches on the mammalian heart of the cat in which the main stem of the auriculo-ventricular bundle had been cut, confirmed the findings of Erlanger and Hirschfelder, and in addition made important observations upon the conditions which govern the length of the ventricular pause following the stoppage of artificial stimuli applied to the ventricles. He stated that the length of the ventricular pause depended upon the duration and rapidity of stimulation preceding the pause; increase in either the duration or rate of artificial stimuli prolonged the ventricular pause. The excitability of the ventricle to electric stimuli remained unchanged during the ventricular standstill, and he concluded that the processes concerned with impulse formation and with excitability were different.

In cases of complete auriculo-ventricular block it is only rarely that one can discover a predisposing cause for the changes in ventricular frequency. The effect of nervous influences is difficult to eliminate, but cases have been reported where the ventricular rhythm appeared to have been altered by them. In the majority of cases, and in those coming under our observation, no determining causes of the changes in ventricular rate, except those due to exercise and excitement, could be discovered. Investigations upon the effect of mechanical conditions of the circulation upon the ventricular rhythm in animals suffering from auriculo-ventricular block have not yet been carried out; this paper deals with experiments bearing upon this problem.

The heart-lung preparation described by Knowlton and Starling (1912) has been used throughout the research; with this preparation both the systolic and diastolic volumes of the heart may be varied at will by altering the venous inflow and arterial resistance. The intra-ventricular pressure is also changed by any alteration in the arterial resistance. Reflex nervous influences are absent—an essential control in the experiments. Hering (1911) has questioned that electrical excitability can in all cases be regarded as a measure of the natural excitability of the heart; therefore if any changes in ventricular rhythm occurred as a result of altering the mechanical conditions of the circulation, there would be no doubt that they were due to the excitability of the heart under the existing conditions of the experiment.

#### DESCRIPTIONS OF EXPERIMENTS.

In all, eight experiments were performed; the animals were anæsthetised with chloroform and ether after a preliminary injection of morphia; a 2 per cent. solution of chloralose in .9 per cent. NaCl was then given intravenously, the dose being one decigramme per kilogramme body-weight.

In all the experiments, with the exception of No. 1, which was carried out in the usual way, a slight modification of technique enabled us to dispense with hirudin, the blood-clotting being prevented by the use of defibrinated blood.

The actual method is as follows: The heart-lung preparation is carried out as usual until everything is ready for the circulation to be switched over through the artificial resistance. The venous reservoir is filled with defibrinated blood from another dog previously bled for the purpose. Just before the aorta is ligatured, the blood in the heart is allowed to flow out through a side tube attached to the cannula which is inserted in the innominate artery. The

aorta and inferior vena cava are then quickly tied, and defibrinated blood is allowed to flow into the right auricle from the venous reservoir. The side tube in the innominate artery is kept open until the defibrinated blood from the venous reservoir has washed out all the animal's own blood from the heart and lungs; it is then closed, and simultaneously the tube from the innominate cannula to the artificial circulation is unclamped. The whole operation from the time the side tube in the innominate cannula is opened to the time when the artificial circulation is connected up takes about 15 seconds. If the heart is kept too long without a resistance to pump against, it will fail. As an additional safeguard to prevent the formation of clots, the blood may be collected for a few seconds from the side tube distal to the arterial resistance before it enters the venous reservoir. The animal's own blood is defibrinated by whipping, and added to the reservoir later. In one experiment carried out in this way the heart was found to be beating well after five hours.

In the heart block operation clamping of the auriculo-ventricular bundle was performed by the method described by Erlanger and Blackman (1909-10), a modified form of the heart clamp designed by Erlanger (1905) being used. The clamp was inserted into the right auricular appendix and the bundle crushed; the heart was then watched in order to determine whether auriculo-ventricular block had been produced; as a rule two or three attempts were necessary before the operation was successful.

*Methods of recording.*—At first attempts were made to record the mechanical contractions of the auricle and ventricle by connecting the right auricular appendix to a light writing lever and attaching a piston myocardiograph to the ventricle; cardiometer and spring tambour tracings were also taken from the ventricle. The blood-pressure was recorded by means of a Hürthle or mercury manometer. The mechanical disturbance of the ventricles consequent upon the variations in the conditions of the artificial circulation was so great as to render a sure interpretation of the ventricular rhythm uncertain. We therefore turned our attention to recording the heart-beats electro-cardiographically. An Einthoven string galvanometer of the standard pattern made by the Cambridge and Paul Scientific Instrument Company was used. The fibre resistance was 3000 ohms, deflection time less than 0.2 sec., and deflections generally 10 mm. per millivolt. Zinc electrodes bound with bandages impregnated with kaolin and .9 per cent. saline were placed at the root of the neck and the under surface of the centre of the diaphragm. After the heart-lung preparation and the operation for auriculo-ventricular heart block had been completed the chest was closed. Unfortunately some of the curves are marred by the collapse and expansion of the lungs, which altered the position of the heart and the amount of surface in contact with the

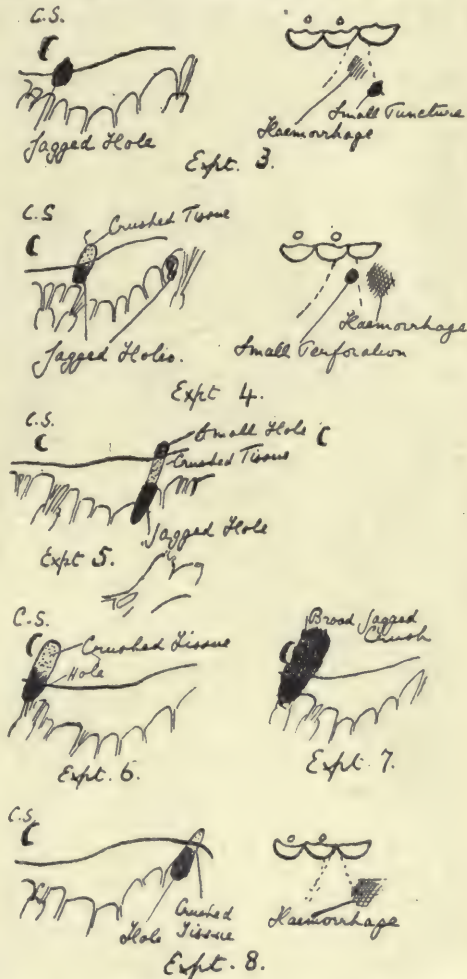


FIG. 7.—Sketches of lesion. About half natural size. C.S. = Coronary sinus.



adjacent tissues; this was more evident with an open pericardium. In later experiments the respirations were stopped during the fall of the photographic plate.

No attempt was made to examine the hearts histologically, as investigations upon the extent of the lesion and resultant effects upon the heart rhythm have been carried out by many workers. Sketches of the lesion were made and are seen in Fig. 7 (p. 3).

#### RESULTS.

*Partial auriculo-ventricular heart block.*—In two experiments (Nos. 5 and 8) partial heart block resulted. In Experiment 5 the block was complete at the beginning of the experiment, but gradually conduction of the auriculo-ventricular bundle improved until 1:1 rhythm with a prolonged P-R interval supervened. Both a large rise in arterial resistance (one reading) and a large increase in venous inflow (two) impaired the conduction of the auriculo-ventricular bundle (Plate I, fig. 1). The actual production of 2:1 auriculo-ventricular block (*b* in Plate I, fig. 1) appears to be due to the fact that every other auricular beat is premature, and the ventricle only responds to the normal rhythm. In this curve we have an example of auricular ectopic beats caused by increased work thrown upon the auricles. In Experiment 8, with the exception of the first record obtained, the rhythm varied between complete and 2:1 heart block; 2:1 auriculo-ventricular block always occurred with the systolic blood-pressure below 130 mm. Hg. (four), and complete heart block with the systolic blood-pressure above 130 mm. Hg. (six). Plate I, fig. 2, shows the effect of raising the mean blood-pressure from 125 to 185 mm. Hg. by changing the arterial resistance. With the higher blood-pressure the auriculo-ventricular block is complete, and the idio-ventricular rhythm is more frequent than the ventricular rate when 2:1 auriculo-ventricular block is present; there appears to be no doubt that the effect is not due to a change in temperature, as the auricular frequency is a little slower in the rhythm with complete auriculo-ventricular block. The effect of intra-ventricular pressure in relation to ventricular frequency will be discussed later.

*Complete auriculo-ventricular block.*—In Experiments 1, 2, 3, 4, 6 and 7, complete auriculo-ventricular block was produced. Mechanical records of the auricle and ventricle were taken in Experiments 1, 2 and 3; the nature of the irregularity, occurring under varying mechanical conditions of the circulation, could not be definitely ascertained and could only be inferred from the arterial pressure curve.

In Experiment 1, a rise in arterial resistance caused ventricular irregularity, as also did a rise in venous inflow; spontaneous disappearance of the new rhythm took place once. A fall in arterial resistance which was previously high caused disappearance of the ventricular irregularity (one).

In Experiment 2, during the first eight alterations of the artificial circulation ventricular irregularities were brought about by a rise (two) and a fall (one) in arterial resistance. A fall in arterial resistance initially high caused the irregularity to disappear (one), and no change in the ventricular rhythm occurred as a result of altering mechanical conditions four times. The succeeding ten alterations comprised a rise in arterial resistance (three), a fall in arterial resistance (five), a rise in venous inflow (one), and a fall in inflow (one); none of these changes were productive of irregularities of the

ventricle with the exception of a single ventricular premature beat, which occurred twice.

In Experiment 3, a rise in arterial resistance produced a ventricular irregularity, which disappeared on the resistance being lowered. An increase in venous inflow also caused ventricular irregularity, but it disappeared spontaneously (Plate I, fig. 3).

Electrocardiograms were taken in Experiments 4, 6 and 7.

In Experiment 4, Plate II, fig. 4, *a*, *b* and *c* show the effect of raising the inflow with a constant arterial resistance; an irregularity occurred, but only the last two beats have been caught. The irregularity disappeared without the venous inflow being reduced. The abnormal complexes in *b* in Plate I, fig. 2, may be due to premature beats of the ventricle arising in the right basal position of the heart, but the curves *a*, *b* and *c* in Plate II, fig. 4, are suggestive of incomplete bundle branch block as described by Wilson and Herrmann (1921). To determine the exact nature of the ventricular irregularity would require more information than we are able to produce, as in these experiments we were only concerned with the possibility of a change of rhythm. It is to be remembered that the leading off electrodes were from the root of the neck and diaphragm, the pericardium was open, so allowing of greater alteration in the heart's axis, and also we have no records of the three standard leads. Our curves, therefore, give no certain indication of the path of the stimulus. In this experiment a rise in arterial resistance to 170 mm. Hg. did not bring about any change in the ventricular rhythm, but when the pericardium was opened a rise in arterial resistance to the same height caused an irregular and more frequent ventricular action (Plate II, fig. 4, *c*). The new rhythm disappeared spontaneously, an infrequent ventricular rhythm supervening. In this experiment we have an analogy to the cases previously described where the idio-ventricular rate is interrupted by a number of ventricular ectopic beats, cessation of the new rhythm being followed by an infrequent ventricular rate. The electrocardiogram in Plate II, fig. 4, *e*, is marred by respiratory effects.

In Experiment 6, an increase in the arterial resistance from 60 to 195 mm. Hg. produced a number of ventricular premature beats apparently arising in the left ventricle. In Plate III, fig. 5, *a*, *b*, shows the effect of an increase in venous inflow with a constant high arterial resistance. Plate III, fig. 5, *c*, *d*, shows the effect of reducing the arterial resistance and venous inflow when they are both initially high. It is possible that complete auriculo-ventricular heart block has given place to 2:1 auriculo-ventricular block in Plate III, fig. 5, *d*, but as the curves were not extended the rhythm is uncertain. The inter-ventricular cycles and the P-R intervals are marked on the curves. The fifth and sixth complexes in Plate III, fig. 5, *b*, at first sight suggest auriculo-ventricular nodal beats, but as there was reason to believe that extrinsic deflections were involved in this experiment, no certain interpretation of the complexes is possible.

In Experiment 7, a more frequent ectopic ventricular rhythm occurred as the result of lowering an initially high arterial resistance. The new rhythm is due to premature ventricular beats probably arising in the left ventricle. A further decrease of arterial resistance to zero, which gave a mean blood-pressure of approximately 40 mm. Hg., caused the premature beats to dis-



appear and the supraventricular rhythm returned, but at a diminished frequency when compared with the initial rate (Plate III, fig. 6, *a*).

#### DISCUSSION OF RESULTS.

In attempting to arrive at an explanation of the attacks of temporary acceleration of ventricular rhythm occurring in subjects with complete heart-block, it must be remembered that division of the auriculo-ventricular bundle does not cut off the sympathetic nerve supply to the ventricles. This has been shown by Cullis (1913) for the cat, and in one experiment we found that after complete division of the bundle in the dog, we could produce acceleration of the idio-ventricular rhythm by stimulating the posterior branch of the *ansa vieussenii*. In this case we obtained an acceleration of ventricular rhythm from 48 to 60 per minute. It must therefore be presumed that this path is open also in patients with heart block, and there can be little doubt that many, if not most, of the cases of temporary acceleration of rhythm incident on exercise and possibly on emotional states may be ascribed to nervous impulses reaching the ventricles along sympathetic channels.

Lutembacher (1921) has shown that adrenalin accelerates the idio-ventricular rate in patients suffering from complete auriculo-ventricular block, but this acceleration is followed by a subsequent slowing of the pulse, so that the tendency to syncope is increased, and the administration of this drug is therefore dangerous in such cases. Adrenalin acts by a direct excitation of the sympathetic nerve-endings or the neuro-muscular junctions, but the course of events observed after its absorption is very similar to that we have described as occurring in man at the beginning of this paper.

There is, however, a further possible explanation which must be considered before ascribing all such alterations of rhythm to sympathetic influence. Ludwig and Luchsinger (1881), working on the isolated frog's ventricle, and Biedermann (1884) on the snail's heart, found that a rise in intra-ventricular pressure increased the frequency of beats, and Biedermann states that in the snail's heart this increased frequency was attended with a decrease in the systolic volume of the heart. In the mammalian heart Hering (1900) has shown that a sudden rise of ventricular pressure may give rise to ventricular premature beats. This could only be explained by ascribing a direct excitatory effect to the increased tension on the ventricular muscle, or, at any rate in the frog, to that part of the ventricle which was initiating the spontaneous beat. We know that in the isolated mammalian heart a rise of intra-ventricular tension, or indeed any other mechanical alteration in the conditions of the circulation, causes no change in cardiac rhythm, the sole means of altering the cardiac rhythm being by changing the temperature of the pace-maker. It seems possible that although the pace-maker in the sino-auricular node is insusceptible to changes in intra-auricular tension, this may be due to some structural arrangements preventing its constituent fibres being stretched by a rise of intra-auricular tension, or by increase of inflow into the auricles. When, after division of the auriculo-ventricular bundle, the ventricle is beating with its own rhythm, initiated either in the auriculo-ventricular node in the bundle itself or in other parts of the ventricular muscle, we might find a direct reaction of such vicarious pace-

makers to increased intra-ventricular tension or to increased volume, or stretching of the ventricles similar to that described by Ludwig and Luchsinger in the frog's ventricle. At any rate, the possibility had to be tested experimentally, since any such reaction of the ventricular muscle might be a contributory cause in the acceleration of ventricular rate observed in cases of complete heart-block as a result of exercise, *i. e.* under conditions where there will be an increased intra-ventricular pressure due to the rise of arterial pressure and possibly an increased ventricular volume.

In our experiments all intervention of nervous impulses was excluded by the conditions of the preparation itself, but in a considerable proportion of them we obtained a quickening of ventricular rhythm and extra beats, as a result of mechanical changes in the circulation. As a rule these could be evoked either by a considerable rise in the arterial resistance or by increasing the venous inflow into the heart. Both these conditions would cause a rise in the intra-ventricular pressure, as well as dilatation and stretching of ventricular muscle. But the fact that they were more easily evoked after the pericardium was freely open seems to indicate that the latter factor, *i. e.* the stretching of the ventricular muscle, was the more important of the two. Normally the pericardium limits the dilatation of the heart, and opening the pericardium, while rendering the heart more efficient for a time, brings this about by allowing increased dilatation and therefore increased length of cardiac muscle-fibre. Our experiments do not, however, permit us to accept this conclusion without reserve. They were all carried out within a short time of the division of the auriculo-ventricular bundle, *i. e.* there was a recent injury of this bundle and of the tissues in its vicinity, which might act as a source of irritation, and which might be susceptible of further tearing and damage when the pressure or filling was suddenly raised and the walls of the heart considerably dilated.

Lewis (1920) says: "It is possible that transient intensification of an inflammatory process in the bundle may sometimes provoke the attacks; an increase of pressure in the blood-vessels or lymph spaces of this region, by altering the tissue tensions, might abolish conduction in a bundle in which it was already precarious." We have shown that this is actually what happened in one of our cases of incomplete heart block, where rise of arterial pressure changed a 2:1 rhythm into complete block, but at the same time seemed to act as a direct excitation of the lesion, since the idio-ventricular rhythm was somewhat greater (78 per minute) than the previous 2:1 rhythm (60 per minute). The fact that in the experiments here recorded the accelerating influence on the ventricles of increased pressure or filling was most marked at the beginning of the experiments, and indeed often passed off while the pressure was still high, seems to indicate that the essential feature was not the increased pressure or stretching of the sound ventricular muscle, but the irritation due to further tearing or injury at the site of the recent lesion. We are thus probably dealing with a condition resembling some active progressive inflammatory lesion in the bundle. We regard this as the most probable explanation of our results, but we cannot exclude the possibility of a direct excitation of sound ventricular tissue until the experiments have been repeated, leaving some weeks between the division of the bundle and the investigation of effects of rise of pressure. We hope to carry out such experiments later.



## CONCLUSIONS.

(1) In confirmation of other observers we have found that after complete division of the auriculo-ventricular bundle, acceleration of the idio-ventricular rhythm can be evoked by stimulation of the cardio-sympathetic nerves.

(2) In the heart separated altogether from nervous impulses, as in the heart-lung preparation, a rise of arterial pressure, or increased inflow into the heart, frequently causes increase in the idio-ventricular rhythm after complete division of the auriculo-ventricular bundle.

(3) It cannot yet be decided whether this result is due to the stimulating effects of increased tension or increased stretching of the ventricular wall, or to a direct irritant effect of the stretching on the recent lesion in the neighbourhood of the auriculo-ventricular bundle. Reasons are given for believing that the latter is the more likely explanation.

(4) After partial division of the auriculo-ventricular bundle, a rise of arterial pressure or increased filling may cause the block to become complete, the block becoming once more incomplete when the pressure or filling is reduced to its previous value.

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FIG. 5.—Exp. 6.

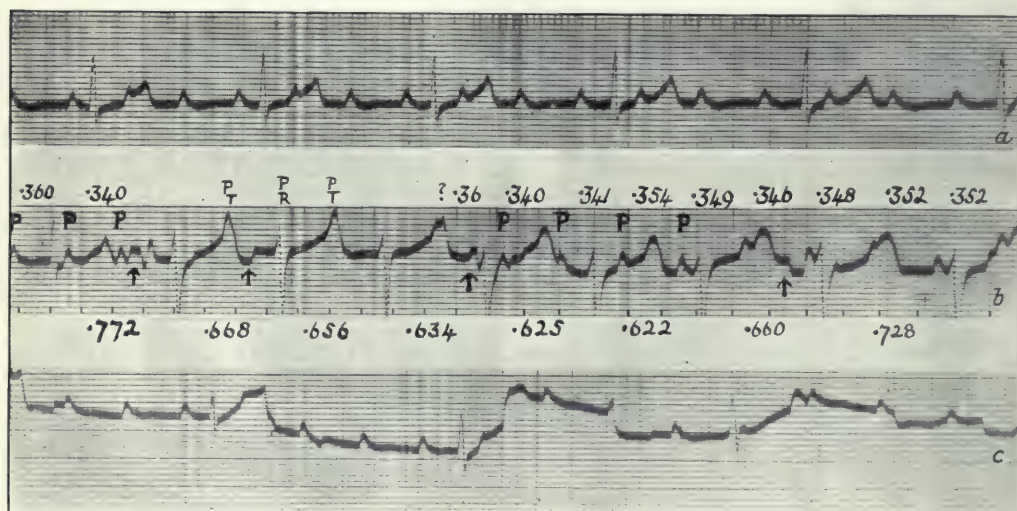


FIG. 6.—Exp. 7.





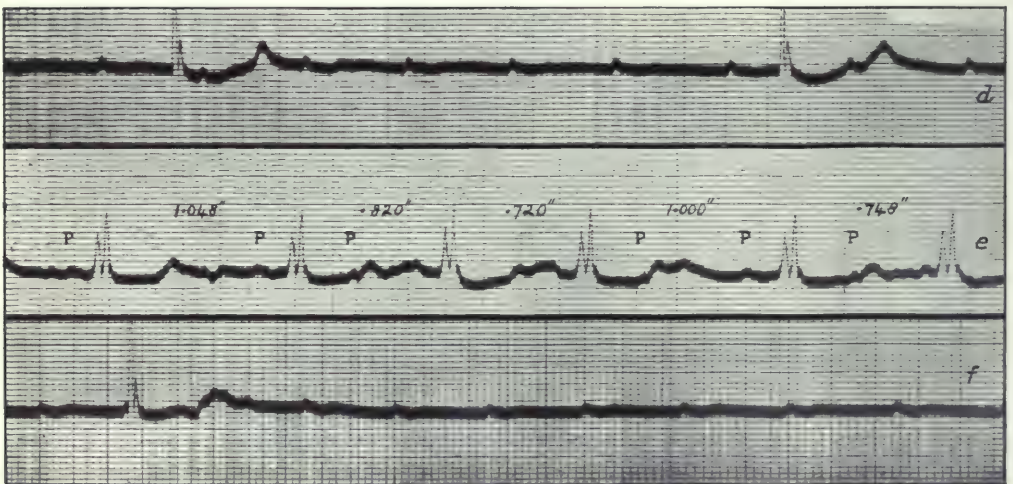
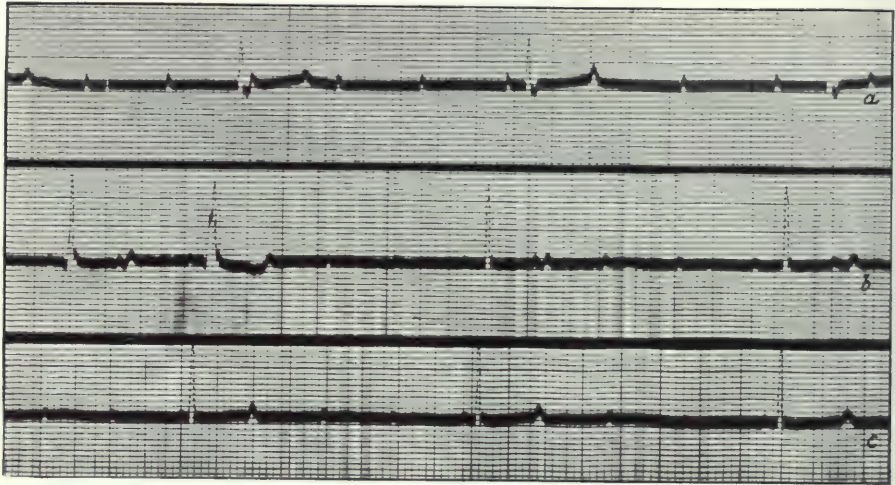


FIG. 4.—Exp. 4.





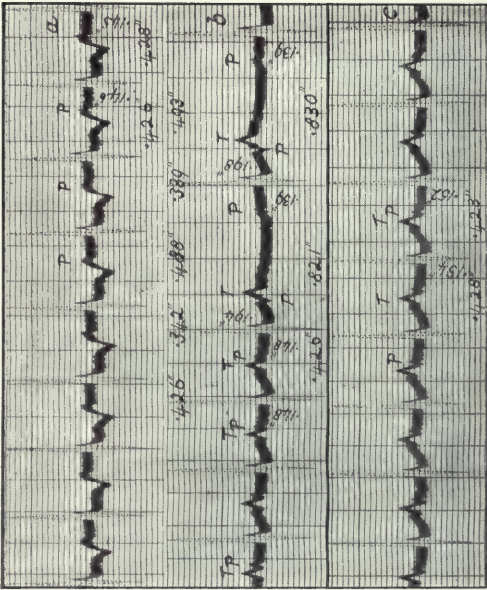


FIG. 1.—Exp. 5.

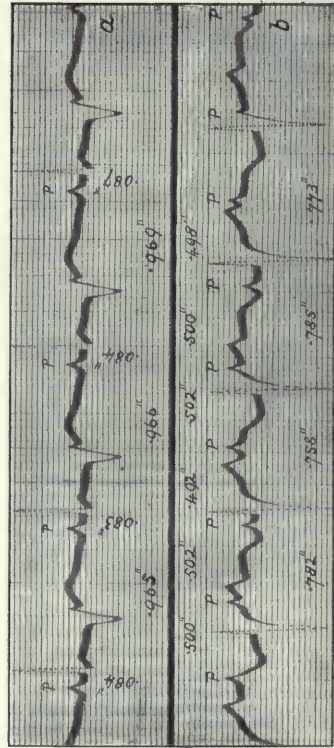


FIG. 2.—Exp. 8.

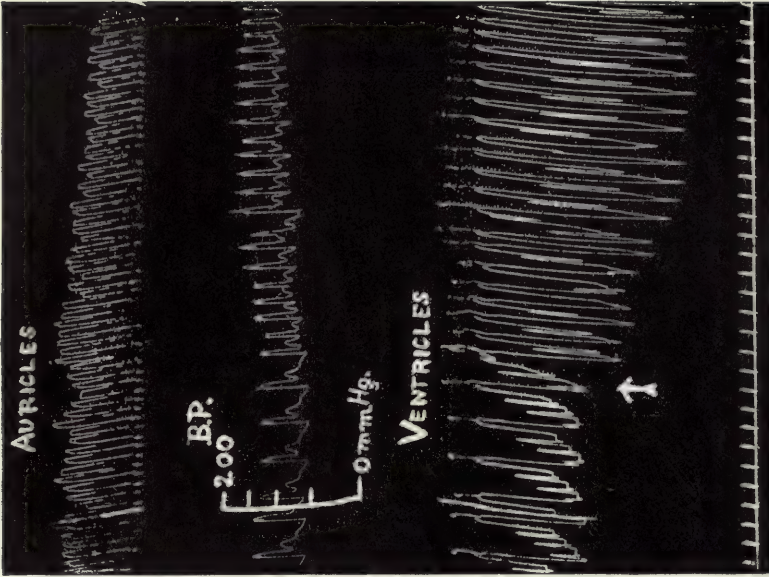


FIG. 3.—Exp. 3.

## EXPLANATION OF PLATES I-III, FIGS. 1-6.

PLATE I, FIG. 1.—Exp. 5. 6.5. '21. Dog, 7.0 kilo. Chloralose. Partial auriculo-ventricular block. Pericardium intact. Effect of raising the arterial resistance :

	O.P.	A.R.	Auricular rate per min.	Ventricular rate per min.	P-R interval.	Temp.
(a)	300	10	140	140	146"	35.0° C.
(b)	170	200	140	140-70	148"	35.0° C.
(c)	170	200	140	140	153"	35.0° C.

(O.P. = Output in c.c. per minute. A.R. = Arterial resistance. Time in this and other tracings,  $\frac{1}{5}$ " and  $\frac{1}{15}$ ".)

PLATE I, FIG. 2.—Exp. 8. 13.6. '21. Dog, 6.6 kilo. Chloralose. Partial auriculo-ventricular block. Pericardium open. Effect of raising the arterial resistance; the inflow was not altered.

	O.P.	M.B.P.	Auricular rate per min.	Ventricular rate.	P-R interval.	Temp.
(a)	217	125	124	62	126"	34.5° C.
	—	185	120	78	complete block	34.5° C.

(M.B.P. = Mean blood-pressure.)

PLATE I, FIG. 3.—Exp. 3. 29.4. '21. Dog, 7.0 kilo. Chloralose. Complete auriculo-ventricular block.

Top line auricle (systole downwards). Middle line B.P. (scale divisions 50 mm. Hg.). Bottom line ventricular movements (systole downwards). Time 1". Effect of increasing the venous inflow at arrow from 180 to 333 c.c. per minute with arterial resistance constant. Temp. 32.5° C.

PLATE II, FIG. 4.—Exp. 4. 2.5. '21. Dog, 7.0 kilo. Chloralose. Complete auriculo-ventricular heart block. Pericardium half open. Effect of increasing the venous inflow (a-c)

	O.P.	A.R.	Auricular rate per min.	Ventricular rate per min.	Temp.
(a)	215	60	120	39.5	33.5° C.
(b)	830	60	150	37.5	34.2° C.
(c)	—	60	150	38.5 (approx.)	34.2° C.

Effect of a rise in arterial resistance immediately after the pericardium had been fully opened (d-f) :

	O.P.	A.R.	Auricular rate per min.	Ventricular rate per min.	Temp.
(d)	215	66	106	18	33.5° C.
(e)	—	170	118	72 (approx.)	—
(f)	—	170	120	—	33.5° C.

PLATE III, FIG. 5.—Exp. 6. 10.6. '21. Dog, 8.4 kilo. Chloralose. Complete auriculo-ventricular heart block. Pericardium open.

(a) Small venous inflow: A.R. 170 mm. Hg.; aur. rate 96; temp. 31° C.

(b) Effect of increasing the venous inflow to a maximum: A.R. 170 mm. Hg.; aur. rate 100; temp. 31° C.

Effect of reducing both the arterial resistance and venous inflow when both are initially high:

	O.P. per min.	S.B.P.	Auricular rate.	Ventricular rate.	Temp.
(c)	660	140	100	Irregular	31.5° C.
(d)	86	80	83	41.5	31.0° C.

PLATE III, FIG. 6.—Exp. 7. 10.6. '21. Bitch, 7.8 kilo. Chloralose. Pericardium open. Complete auriculo-ventricular block. Effect of decreasing the arterial resistance:

	O.P.	A.R.	Auricular rate per min.	Ventricular rate per min.	Temp.
(a)	375	175	172	55	36.0° C.
(b)	350	90	172	92	36.0° C.
(c)	—	0	158	38.5 (approx.)	36.0° C.

(b) and (c) are marred by deflections due to the artificial respiration, shown by arrows.



# THE IMPORTANCE OF THE INORGANIC CONSTITUENTS OF THE FOOD IN NUTRITIONAL DISORDERS. I: RICKETS IN PIGS.

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RICKETS is very prevalent among pigs kept in confinement. In some districts the incidence of the disease in litters born in the beginning of winter is as high as 50 per cent. It is thus a cause of very considerable loss to the agricultural community and to the country.

Whether the ætiology of the condition is similar to that of human rickets is not definitely established. The chief gross signs of the disease are, in order of sequence of time, rough lustreless appearance of coat with excessive growth of hair, loss of appetite, slowing of rate of growth, lethargy, difficulty in locomotion, as shown first by a stiff, stilted gait. Later there follows loss of power of the hind legs, tendency to fracture of the ribs and deformities of the long bones. Slowing of the rate of growth usually affects the head less than the rest of the body, so that in the late stages the head often appears abnormally large.

The experiments described here were carried out in the course of an investigation undertaken on account of the economic importance of the subject, to determine what conditions of housing or feeding favour the onset of the disease. Some of the results obtained which seem of interest in connection with recent work on experimental rickets are presented here.

## METHODS AND NATURE OF INVESTIGATION.

A feeding experiment carried out here in 1919-20 showed that young pigs in confinement on a diet of wheat offal, grains and potatoes develop the rickets syndrome as outlined above. It was thus possible to arrange a basal diet of these feeding stuffs which would with a degree of certainty produce the condition, and, by the addition of various substances to this diet, to determine what factors prevented the occurrence of the disease.

A consideration of the nature of wheat offal, grains and potatoes suggests (1) deficiency of "fat soluble A," and (2) a lack of correspondence between the mineral matter of the food and the mineral requirements of the animal.

A series of experiments were carried out, in each of which, in a control group of animals, the supposed deficiency of the basal ration in both "fat soluble A" and mineral matter was roughly adjusted, and in the experimental group the deficiency in either "fat soluble A" or mineral matter was allowed to remain, so that the influence of each of these factors in preventing the disease was tested separately. "Fat soluble A" was added in the form of cod-liver oil. The salt mixture used to adjust the mineral deficiency was a rough

imitation of the ash of milk with either additional calcium, to make good the marked calcium deficiency of the basal diet and sodium hydrate to reduce the excessive acidity, or of a calcium-rich mixture of salts supplied in a separate box to which the animal had access. The details are given with the experimental data. Water soluble C was added to all the rations in the form of either lemon juice or swede turnip to avoid the possible complication of deficiency of this factor, though, as a matter of fact, the result of a series of experiments which we conducted in the course of the investigation seem to indicate that the pig is very little susceptible to a deficiency of this vitamin.

In this communication four experiments are briefly described, two showing the influence of deficiency of mineral matter, and two the influence of deficiency of fat soluble A vitamin.

#### EXPERIMENTAL DATA:

##### INFLUENCE OF INORGANIC CONSTITUENTS.

*Experiment 1.*—Two groups each of three pigs two months old were housed under identical conditions in adjoining pens. The pens had an open-air run, with a brick floor. The groups were comparable as to sex and litter, *i. e.* each pig in one group was balanced by a pig of the same sex out of the same litter in the other group.

The basal diet was as follows:

	Proportion.
Oatmeal . . . . .	100
Bran . . . . .	125
Blood meal . . . . .	25
Swede turnip . . . . .	75
Potatoes . . . . .	37·5

Marmite, 20 gm. to each group per day.

Cod-liver oil, 30 c.c. to each group per day.

To reduce the proportion of protein in the diet as the animals increased in age, as is usually done in practice in feeding stock, blood meal was replaced by stages by maize meal. Water was always available. The animals were allowed to eat to their appetites.

One group received only the ration. To the other group there was added per pig per day 2 gm. calcium phosphate and 0·25 gm. each of magnesium sulphate, potassium carbonate, ferric chloride and sodium chloride. In addition to the small quantities of inorganic matter added to the food there was always in the pen a box, which was kept full of a mixture of salts, consisting chiefly of chalk, bone meal, rock salt and coal ashes. The mixture was eaten by the animals, and had to be renewed from time to time.

The feeding was continued for four months.

*Results.*—The animals of the group receiving the salts showed normal growth and no signs of disease. Those of the group receiving only the basal ration began to show signs of malnutrition within the first thirty days. There was lack of lustre of the hair, decreased appetite and slower rate of growth. By the end of the second month the animals had become lethargic, and when forced to move, their gait was stiff and stilted. In the third month deformities of the limbs became evident.



The comparative rates of growth are shown by the increase in weights given in the following table :

TABLE I.—*Increase in Weights.*

	Initial weight. Kilos.	Weight after 124 days. Kilos.	
No. 36 . . .	13.2	35.5	} Ration only.
No. 37 . . .	11.3	43.0	
No. 27 . . .	19.4	59.7	
No. 40 . . .	11.3	66.0	} Ration plus salts.
No. 31 . . .	11.9	73.2	
No. 33 . . .	19.7	85.0	

The average gain per pig per day was in the "ration only" group 0.25 and in the "ration plus salts" group 0.49 kilos.

On the 124th day No. 27 and No. 31—corresponding animals in the two groups—were killed and the percentage of calcium and phosphorus in the eighth rib and femur estimated :

TABLE II.—*Showing Percentage of Ca and P in Ribs and Femur.*

	Ribs.		Femur.		
	CaO.	PO.	CaO.	PO.	
No. 27 . . .	17.1	14.2	29.6	22.8	No extra salts.
No. 31 . . .	20.6	16.5	33.4	25.7	Calcium-rich salts.

Microscopic examination of the bones of No. 27 as compared with No. 31 showed some irregularity of the epiphysial line, an excess of osteoid tissue and disorganisation of the zone of proliferation in the cartilage.

*Experiment 2.*—In this experiment the basal diet was similar in nature to that on which Mellanby (1918) produced rickets in puppies, except that the fat was removed from the milk. Oatmeal and rice were fed in equal proportions according to appetite with an addition of 800 c.c. machine-skimmed milk per animal, *i. e.* about 80 c.c. per kilo weight of the animal at the beginning of the experiment, and 10 grm. sodium chloride per pig per day.

Four groups, each of four seven-week-old newly weaned pigs, were used. The groups as before were comparable with each other as to sex and litter, and the housing conditions were identical.

In comparison with the milk of either the bitch or the sow, Mellanby's basal diet is very poor in protein. Bitch's milk contains roughly, per cent., protein 10, fat 11, carbohydrate 3, and for the sow the respective figures are 7, 5 and 3. All the food-stuffs of the basal diet contain a much lower percentage of protein in proportion to the other energy-yielding constituents than that present in the milk of the species experimented on. To see whether deficiency of protein has any influence, additional protein in the form of blood meal, *i. e.* dried blood, was added to the basal ration of two of the groups to the extent of one-sixteenth of the total weight of oatmeal and rice given.

To the food of one of the two groups receiving the ration only and one of the two receiving the ration plus extra protein there was added per pig per day 20 grm. of the salt mixture used in Experiment 1 plus 5 grm. calcium

carbonate. In addition there was added to every 6 pounds of mixture of rice and meal 80 c.c. of 10 per cent. sodium hydrate—the approximate amount necessary to neutralise the excessive acidity of the ration.

The diets were therefore as follows :

Group I.	Group II.	Group III.	Group IV.
Ration only .	Ration + salts .	Ration + protein .	Ration + protein + salts.

Water was always before the animals but no additional mineral matter was provided.

*Results.*—During the first fortnight the rate of growth of the two groups receiving the extra protein exceeded that of the other two groups. During the second fortnight the signs of malnutrition noted in Experiment 1 became evident in Group III, and one of the animals died on the thirty-third day of the experiment. The *post-mortem* examination showed anæmia and patches of inflammation and adhesions in the pleura of the lungs. In the third fortnight the signs of disease noted in Group III appeared in Group I. Towards the end of the second month two of the three remaining animals of Group III were hardly able to walk and were reluctant even to stand. The remaining animal of the group had acquired the habit of drinking the urine of its companion. It was the last to show signs of disease.

The following table shows the average gains in weight in the different groups during the first 30 days. Owing to the death in Group III the averages for the second month during which the experiment was run are not comparable.

TABLE III.—Average Gains in Weight in Different Groups.

Groups.	Initial weight.	Gain in weight.	
	Kilos.	1st 15 days.	2nd 15 days.
I. Ration only . . . . .	9·8	3·2	3·4
II. Ration + salts . . . . .	9·8	3·1	4·4
III. Ration + protein . . . . .	9·7	3·6	2·5
IV. Ration + protein + salts . . . . .	9·8	3·5	4·9

On the sixty-ninth day one of the animals of Group III was killed. The following table shows the percentage calcium in the eighth rib compared with that found in a healthy animal of the same age.

TABLE IV.—Showing Percentage Composition of Dried Rib (Eighth).

Organic matter.	Ash.	CaO.	PO.
58·3 .	41·7 .	21·7 .	17·3 No. 99 of Group III.
46·6 .	53·4 .	28·0 .	22·0 Normal animal same age.

Microscopic examination of the bones showed the same pathological characters as those found in Experiment 1, but the condition was less advanced.

No sign of rickets appeared in either of the groups receiving the extra salts. The signs of the disease usually appear first, as Mellanby (1918) has pointed out, in the most rapidly growing animals. In Groups II and IV the rate of growth was rather above the normal. One of the animals in Group II



during the last ten days of the experiment increased in weight 0·97 kilos per day and one in Group IV 0·82 kilos. In these groups there was no sign of malnutrition, though the diet, with the exception of the inorganic matter, was exactly similar to that of Groups I and III, where the gross evidence of the disease appeared within thirty days.

#### INFLUENCE OF FAT SOLUBLE A (COD-LIVER OIL).

*Experiment 3.*—In this experiment ample inorganic matter was given to both groups, and one group was given additional fat soluble A in the form of cod-liver oil, so that the influence of a relative deficiency of "fat soluble A" was determined in the other group.

Two groups each of three animals all out of the same litter were given the following ration :

	Proportion.
Oatmeal . . . . .	100
White bread (replaced after first month by maize) . . . . .	200
Cod muscle . . . . .	200
Swede turnip . . . . .	100
Marmite, 20 grm. per day.	
Salt mixture as in Experiment 1.	

Access was allowed to water and to a salt box as in Experiment 1.

Cod muscle was used as the supply of protein, because it contains no fat soluble A (Medical Research Committee, 1919).

To one group was given 10 c.c., rising to 20 c.c. cod-liver oil, and to the other 10 grm., rising to 20 grm. rendered lard, which is stated to contain little or no fat soluble A.

*Results.*—So soon as the experiment commenced all the animals of both groups began to suffer from diarrhœa. The condition was more marked in the lard group. One of the animals in this group (No. 23) developed distension of the abdomen and a rough "staring" coat—a sure indication of malnutrition. Boiling the fish produced no improvement. At the end of the first month maize meal was substituted for bread and the diarrhœa stopped in both groups within 48 hours.

During the subsequent period of the experiment, which was continued for 132 days, no signs of disease appeared in either of the groups. At the close of the experiment, however, the general appearance of the cod-liver oil group was better than that of the lard group, in so far as the coats were more silky and glossy—a sure sign of health—and the increase in weight was greater.

TABLE V.—*Showing Increase in Weight in Kilos.*

No.	Beginning of experiment.	105 days.	132 days.	
No. 14 . . . . .	11·7 . . . . .	69·0 . . . . .	80·8 . . . . .	} Cod liver oil.
„ 15 . . . . .	12·6 . . . . .	70·0 . . . . .	— . . . . .	
„ 16 . . . . .	13·0 . . . . .	75·1 . . . . .	95·8 . . . . .	
„ 17 . . . . .	10·9 . . . . .	61·3 . . . . .	69·5 . . . . .	} Lard.
„ 20 . . . . .	12·1 . . . . .	60·8 . . . . .	70·7 . . . . .	
„ 23 . . . . .	13·5 . . . . .	68·2 . . . . .	83·2 . . . . .	

Table V shows the respective increase in weight (1) on the 105th day, when one of the cod-liver oil group had to be killed owing to its having received an accidental traumatic injury and (2) on the 132nd day—the end of the experiment.

No. 23 of the lard group, the animal which had shown the signs of malnutrition in the earlier part of the experiment, was continued on the diet until the 140th day, when it was killed. During the last 30 days of this period it had shown an average increase in weight of 0.61 kilos per day. The post-mortem examination showed no abnormality. The epiphysial line was regular and clearly defined, and the bones were of the usual degree of hardness. No. 20 was also killed and showed nothing abnormal in the post-mortem examination. No. 17, a sow, was kept for breeding purposes. She was turned out to graze and mated 34 days after the close of the experiment and had a litter of eight healthy pigs.

*Experiment 4.*—Two groups each of four seven-week-old pigs comparable as to sex and litter and in similar housing were given the following basal diet :

	Proportion.
Bran . . . . .	100
Oats (crushed) . . . . .	100
Blood meal . . . . .	8
Salt mixture as in Experiment 2.	
50 c.c. lemon juice per group.	

The proportion of blood meal was reduced by stages as the animal increased in weight and entirely omitted at the end of the third 30 days.

There was added to one group 10 c.c. cod-liver oil and to the other 10 c.c. linseed oil per pig per day. The linseed oil, which is deficient in A factor (Medical Research Committee, 1919), was added to balance as far as possible the caloric value and the physical properties of the cod-liver oil.

The experiment was continued for 120 days.

*Results.*—None of the animals throve during the first month. The gains in weight were below the normal and the coats had not the silky, glossy appearance that denotes perfect health in the pig. The diet was probably too harsh for newly-weaned pigs. Both groups improved in condition as the experiment proceeded. No signs of disease appeared in any of the animals.

During the first 60 days the linseed oil group grew faster than the cod-liver oil group. Thereafter the reverse was the case. The following table shows the increase in weight per pig during the successive periods of 30 days :

TABLE VI.—Average Increase in Weight per Pig per Day in Kilos.

	1-30.	31-60.	61-90.	91-120 days.
Cod-liver oil group	·086	·238	·434	·538
Linseed oil group . .	·134	·266	·403	·453

In Experiments 1 and 2 definite signs of the disease appeared during the second month. As this experiment had been continued for 120 days and the animals had passed the stage when rickets usually develops and all appeared in perfect health, the experiment so far as rickets is concerned was considered finished.



One animal out of each group was killed and examined. No abnormality was discovered in either. The percentages of calcium and phosphorus in the femur are given in the following table:

TABLE VII.—*Showing percentage of Ca and P in Femur.*

	CaO.		P <sub>2</sub> O <sub>5</sub> .	
No. 106	34.68	.	26.24	Cod liver oil.
No. 108	34.21	.	26.58	Linseed oil.

#### SUMMARY OF RESULTS.

In Experiment 1, on a diet in which the energy-yielding constituents are derived chiefly from bran and oatmeal, and which contained an abundance of the three well-known accessory factors, the signs of rickets appeared during the second month. In animals of the same sex and litter, housed under identical conditions and receiving the same diet, but with the addition of a mixture of inorganic material consisting chiefly of calcium salts, no signs of the disease were apparent at the end of four months, by which time the animals had passed the stage where the disease usually appears.

In Experiment 2, on a diet of oatmeal and rice with 80 c.c. skimmed milk and 1 grm. sodium chloride per day per kilo weight of the animal at the beginning of the feeding period, the signs of the disease developed and were well marked by the end of the second month. The addition of a calcium-rich salt mixture to a control group under the same condition of housing and feeding prevented the development of the disease.

In the group receiving the protein-rich diet the growth at first was more rapid and the pathological condition developed earlier. This is in accordance with Mellanby's (1918) observation on rickets in puppies, that the most rapidly growing puppies are most susceptible to the disease.

In Experiments 3 and 4 the diets were purposely designed to contain a minimum of accessory factor "fat soluble A," and certainly contained no more of this than is present in the rations of animals where the disease is occurring in practice. Both the experimental and the control groups had, however, a *luxus* consumption of calcium-rich mixture of salts. Though the monotonous diet was continued in the one case for 120 days and in the other for 140 days, the rickets syndrome did not appear.

In both of these experiments, though the groups receiving the diet deficient in fat soluble A did not develop rickets, the growth there was less vigorous than in the group receiving the cod-liver oil. Further, in Experiment 3 the health of the animals, as evidenced by the condition of the coat and their activity and liveliness, was better in the group receiving cod-liver oil than in the one receiving the lard.

#### DISCUSSION OF RESULTS.

In connection with this investigation, papers will shortly be published dealing with the mineral requirements of growing animals, and the physiological importance of the ratio of the inorganic constituents of food-stuffs to each other. The results presented here need therefore only be discussed briefly.

Grains, and food-stuffs derived from them, are, in general, characterised by a deficiency of calcium, and by the fact that the ratio of magnesium to calcium, of phosphorus to calcium and of potassium to sodium is very high. The percentages of these minerals in the dry matter of the chief feeding stuffs used in the present experiments are as follows (Forbes, 1913):

	K.	Na.	Ca.	Mg.	P.
Bran . . .	1.46	0.22	0.14	0.59	1.23
Oats . . .	0.46	0.18	0.11	0.13	0.43
Rice . . .	0.32	0.04	0.01	0.03	0.10
Potatoes . .	1.55	0.06	0.03	0.33	0.27

It may be assumed that the proportions in which these minerals are present in milk are those best suited for the growing animal. In the ash of the sow's milk, the percentages as quoted in König's tables (1889) are as follows:

K.	Na.	Ca.	Mg.	P.
6.22	6.73	39.22	1.77	37.21

Compared with this composition of ash, there was in the basal diets used here a deficiency of calcium, and a relative excess of magnesium, potassium and phosphorus. The addition of calcium-rich salts to the ration used in these experiments would tend to correct the mineral deficiency.

The beneficial effects of calcium for farm stock suffering from nutritional disorders affecting the bones has long been known. A review of the literature to 1912 is given by Hutyra and Marek (1913).

With regard to work on the importance of the proportions in which the inorganic constituents are present, Ingle (1908) has pointed out the evil effects that may follow a prolonged diet in which there is a great excess of phosphorus in proportion to calcium, and has suggested that the well-known "bran disease," or "miller's horse rickets," is due to this cause. Zuntz (1912) has shown that certain fodders that cause bone-lesions in horses are injurious in proportion to the excess of potassium to sodium in the ash.

Hart and McCollum, with their co-workers, in the course of their valuable work on deficiencies of the different grains have shown that the deficiency of grains in mineral matter is so great that a diet consisting exclusively of grains will not support growth, even though any other deficiencies that may be present are made good. Hart, Miller and McCollum (1916) produced a pathological condition in pigs which, from the description given, appears to be similar to that which we have been investigating. They indicate that three factors may be involved in the production of the disease, viz. (1) deficiency of mineral matter, (2) deficiency of fat soluble A, and (3) toxicity of wheat products. In our experiments the condition did not occur when the amount and ratios of the different inorganic constituents were adjusted, and we are inclined to believe that the frequently noted toxic influence of wheat offal is due to the composition of its ash, which, with other deficiencies, shows a marked excess of phosphorus to calcium. Their own results seem to support this view. They state—"where alfalfa constituted 20 to 25 per cent. of the ration and middlings 20 per cent., the toxicity of the middlings was overcome, the inadequacy of the



grains disappeared, and the animals remained sound and vigorous"; and again, "with commercial meat scraps as supplements to a corn, middlings, oats, oil mixture as previously described, we have also had splendid success in obtaining normal growth and sustained vigour." Both these additions to the diet tended to adjust the mineral matter. The meat scraps used by them contained 6.5 per cent. calcium, and alfalfa is very rich in calcium as compared with phosphorus. Forbes (1913) gives the percentage composition of the dry substance of alfalfa as—

K.	Na.	Ca.	Mg.	P.
0.83	0.49	1.13	0.40	0.24

An abundant supply of calcium of course does not correct the mineral deficiency in every case. Prominence is given to it here because, with regard to mineral matter, deficiency of calcium was the marked feature in the diets used in the experiments and was probably the chief factor in the production of the disease. Shipley, Park, McCollum and Simmonds (1921) have shown the importance of phosphorus in the prevention of rickets, and Sherman and Pappenheimer (1921) have shown that in deficiency of both calcium and phosphorus osteoporosis occurs; with the addition of calcium lactate rickets occurs, and with the further addition of potassium phosphate rickets is prevented. It is most probable that the presence of a sufficient amount of any essential inorganic constituent need not necessarily prevent rickets. To ensure normal growth and health there must be not only a sufficiency of each of the essential inorganic constituents, but the ratios of these to each other must be adjusted to the needs of the animal, or at least must not show such a wide divergence from the physiological optimum that the excess of any of them is greater than the animal can deal with without upsetting the normal metabolic processes. Unfortunately, exact information as to what excess of the different minerals can be tolerated is not available.

The influence of fat soluble A can be briefly discussed. It is obvious that an abundance or relative deficiency of it need not necessarily prevent or produce rickets. The results give no support to the suggestion of the Cambridge school that rickets is due to a deficiency of an accessory factor probably identical with fat soluble A. They are in an agreement rather with the work of Noël Paton and Watson (1921), which shows little or no correlation between rickets and any substance associated with animal fats.

It has been already shown by Zilva, Golding, Drummond and Coward (1921) that pigs can continue to grow for at least 70 days on a diet with a low content in fat soluble A. In these present experiments the small amount of fat soluble A in the diet used has enabled growth to proceed over a period of 140 days, during which time the animals increased in weight by over 500 per cent. On the other hand the well-recognised value of cod-liver oil compared with either linseed oil or lard in maintaining health and promoting growth is shown even in the pig, which evidently is not so susceptible to deficiency of fat soluble A as the rat. It is possible that the beneficial effects of certain fats may in part at least be associated with their influence on the assimilation and utilisation of inorganic substances. That is being investigated here.

Since the above paper was prepared there has appeared a communication by Korenchevsky (1921) dealing with the influence of deficiency of calcium and of fat soluble A in producing rickets in rats. He shows that rickets occurs most readily when the diet is deficient in both calcium and fat soluble A. The deficiency of calcium alone, however, produces a change in the skeleton resembling rickets.

## NOTES.

(1) The experiments described here and the conclusions drawn from them were dealt with in a paper read to the Agricultural Section of the British Association Meetings at Edinburgh, September 13th, 1921.

(2) We wish to express our indebtedness to Dr. G. M. Duncan for carrying out the pathological work in connection with these experiments, and to Dr. Marion B. Richards and Mr. A. D. Husband for the chemical analysis of the bones.

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## A COMPARATIVE STUDY OF NORMAL AND MALIGNANT TISSUES GROWN IN ARTIFICIAL CULTURE.

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ONE of the easiest methods to obtain comparative biochemical information as to the metabolism of both normal and malignant cells would be to treat such cells as bacteria and grow them in suitable media outside the body. The present work is an attempt to study cells from this point of view and to arrive at a satisfactory artificial medium in which they may be cultured indefinitely. Carrel and Burrows (1912) and others have shown that cells may be grown for many generations in certain media. The great majority, however, of the American workers have used plasma as a culture medium either alone or with the addition of various extracts. Apart from the technical difficulties of growing tissues in such media, plasma is very unsatisfactory when accurate biochemical information is required. Slight differences in various samples of plasma often make great differences in growth, and it is at all times very difficult to control. Even for histological study plasma growths leave much to be desired, as it is often found that the plasma retains the stain quite as tenaciously as the cells themselves. Before discussing artificial culture media, it is necessary to consider the features of growth in plasma of embryonic tissues, adult tissues, and tumours.

The whole of our own work has been carried out with either rat or mouse tissues, and mouse tumours in rat plasma. Most of the American workers have used bird plasma, but as it was thought desirable to use a plasma differing as little as possible from that of the animals from which the tissues were taken, rat plasma was chosen as being specially suitable. It has been found possible considerably to simplify the usual technique for obtaining such material. A hollow platinum needle such as is used for the transplantation of tumours is fused into a glass tube some 20 cm. in length and about 5 mm. internal diameter. This is thoroughly coated with paraffin wax by immersion in a bath of equal parts of paraffin of M.P. 45° C. and 56° C. When required for use the tube is lifted out and quickly drained, air being blown through in order to keep the needle clear. The rat is anaesthetised with ether and the thorax quickly opened with aseptic precautions; the heart is then steadied with forceps and the needle thrust into the right ventricle. The blood is allowed to flow into the paraffined tube till the flow slackens. The needle is then withdrawn

and the contents at once run into paraffin-coated sterile glass centrifuge tubes and centrifuged at high speed for three minutes. I have found the type of tube shown in Text-fig. 1 to be the most convenient pattern for the work. The tubes should be thoroughly cooled before the blood is run in and it is best to surround them during centrifuging with ice and salt mixture. The supernatant plasma is removed with a paraffined Pasteur pipette and is again centrifuged in the cold for one or two minutes. This is necessary to separate the blood-platelets as far as possible and so delay clotting. About two-thirds of the resulting plasma is next pipetted off with a paraffined pipette and is placed in a sterile paraffin-coated glass tube, which is kept in a wider tube packed in ice and salt. The tissues to be cultured are removed from the animal with strict aseptic precautions, and are transferred to sterile glass capsules containing a little sterile Ringer's solution. There is no necessity to keep the tissues at 37° C. prior to culture; in fact they may be stored in the ice-chest, and will still give growths after five days. When required for culture the organ selected, *e.g.* heart, is cut up into small pieces with iridectomy knives and the pieces are kept moist by a few drops of Ringer's solution. A piece is placed on a sterile cover-glass, and is then covered with plasma which is added from a sterile paraffined Pasteur pipette. The plasma clots in the space of a minute or two and the cover-glass is then placed over a well slide and is sealed with paraffin wax. It is then incubated at 37° C. In order to reduce the chances of infection to a minimum it is best to carry out the culturing under cover of a glass case, which can be flamed immediately before use. Subculturing is performed by raising the cover-glass by means of a safety-razor blade, cutting carefully round the growth with iridectomy knives, taking especial care to free it from all traces of plasma. The fragment is next washed quickly in sterile Ringer's solution, and divided if necessary, each piece being planted into fresh plasma as already described.



FIG. 1.—Centrifuge tube for collection of plasma.

All tissues do not behave alike when grown in plasma in this way. The differences can best be brought out by a short description of the results with mouse embryo heart, adult mouse heart, and a rapidly growing mouse sarcoma—37 S. of this laboratory. In 24 hours at 37° C. the embryo heart shows growth all round the edges of the original fragment, the growth consisting of both heart-cells and connective tissue. The adult heart shows no growth whatever either of connective-tissue cells or heart-cells, whilst the sarcoma shows first a circular area of liquefaction round the original fragment with extensive growth around this, in the form of a ring (Plate I, fig. 2*a*); such ring-shaped growths are very characteristic of sarcoma grown in rat plasma. These growths have been frequently described and pictured by several authors, but no explanation of this arrangement of the cells has been advanced. We have observed that cultures of this type show an early liquefaction of the fibrin network immediately surrounding the implanted fragment. Contraction of the peripheral portions of the fibrin network will of necessity lead to the circular



arrangement of the growing cells. The embryo heart continues to grow for several days and may beat actively; the adult heart as a rule shows no signs of growth at any time, but occasionally after a lag of about a week may show slight growth in a very small percentage of cases.

The sarcoma begins to degenerate in 48 hours and unless subcultured dies in from two to three days. Provided it is subcultured at intervals, the embryo heart will live and continue to grow for as long as forty days, but growth finally ceases. The sarcoma, as already stated, degenerates very rapidly after the second day. Repeated subculture every day will, however, give vigorous growths for a month or more, when, as in the case of embryo heart, the cultures die out. Inoculations of such repeatedly subcultured growths into mice give tumours in every case, showing the tumour-cells to be alive and unaltered biologically. Such a process of growth as occurs with embryo heart in a plain plasma medium may be considered typical of all normal embryonic tissues, as I have found all those studied to behave in a similar manner.

From the behaviour of the 37 sarcoma it seemed probable that some toxic substance was elaborated which caused the very early degeneration. A number of plasma cultures were accordingly taken, and both plasma and growth were ground up with sterile sand and a very small quantity of distilled water; this emulsion was then centrifuged and the clear supernatant fluid pipetted off. A series of 12 cultures of embryo heart and 37 S. were put up in a plasma medium containing  $\frac{4}{5}$  plasma and  $\frac{1}{5}$  of the 37 S. extract. Along with these cultures a further series of heart and 37 S. cultures were prepared containing  $\frac{4}{5}$  plasma and  $\frac{1}{5}$  distilled water. The embryonic heart grew in every case in both media. The sarcoma grew in every instance in the plasma diluted with distilled water, but failed completely in those fragments immersed in plasma diluted with the extract of 37 S. cultures.

It is evident that 37 S. in culture produces some substance which is toxic to itself and can inhibit growth *in vitro*. This substance is evidently not toxic to heart. Heat at 60°C. for half an hour destroyed its toxicity. Most of the tumours tested showed varying degrees of toxicity but not to the same extent as did 37 S.

It will be observed from these experiments that the various tissues and tumours can be grouped into three divisions, viz. embryonic tissues, which grow vigorously in a plain plasma medium; tumours which also give growth, but not to such an extent as do the embryonic cells; and adult tissues which show little or no growth. This point will have to be discussed in greater detail when considering the growth of tissues in artificial media. The addition of various amino-acids (glycine, alanine, valine, arginine and histidine) to rat plasma gave in most cases a somewhat greater initial growth than was obtained in plain plasma. Microscopic examination of such growths showed that the majority of the cells were undergoing amitotic division, very few mitoses being observed. Plasma rendered hypotonic by the addition of distilled water gave an exactly similar effect. I believe that this represents a degenerative phenomenon, as cultures made in this way die out at an earlier date than do plain plasma cultures, and there seems to be little doubt that the cells are quite unable to use the amino-acids as food

substances—in fact the continued presence of these acids seems to be toxic to the cells.

It is extremely desirable to be able to dispense with plasma as a culture medium in order that the conditions may be more rigidly controlled. Lewis (1920) showed that a certain amount of growth takes place in Locke's solution containing 0·25 per cent. glucose and 10 per cent. of chicken bouillon. Such growths appear to have been poor, however, and probably represent little more than survival of cells. It was found that better growths could be obtained with this Locke-Lewis solution if the calcium chloride and the sodium bicarbonate were steamed separately and added before use. Cultures in such solutions, however, tend to die out at an early date, and cannot be subcultured indefinitely, and are never equal to growths obtained in plasma. After many trials the following solution, the inorganic constituents of which are nearly identical with those of plasma, was found to be satisfactory :

NaCl	.	.	.	0·900
KCl	.	.	.	0·042
NaHCO <sub>3</sub>	.	.	.	0·020
CaCl <sub>2</sub>	.	.	.	0·020
CaH <sub>4</sub> (PO <sub>4</sub> ) <sub>2</sub>	.	.	.	0·010
MgHPO <sub>4</sub>	.	.	.	0·010
H <sub>2</sub> O	.	.	.	100

The salts used in the preparation of this solution must be pure. It will be found best to make up stock solutions of the various salts ten times as strong as the final concentration. Ten c.c. of the NaCl, KCl and MgHPO<sub>4</sub> solutions are then pipetted into a flask and 40 c.c. of distilled water added. The calcium solutions and the bicarbonate are steamed in three separate flasks, and just before use 10 c.c. of each is pipetted into the mixture. The solutions should not be autoclaved. The best results are got by steaming for not more than ten minutes. The finished solution should have a faint bluish opalescence. If a precipitate is formed such as occurs when the fluid is autoclaved it is useless. The pH of the fluid should be 7·4, since growth only appears to occur within very narrow limits of this value. Examination of the fluid so prepared by dark-ground illumination shows very fine particles in active movement. These disappear on the addition of a weak acid, such as acetic acid. In this solution I have obtained as good growths of embryonic tissues as in plasma, for a few generations. The same remarks, however, that were made regarding plain plasma growths apply here, viz. that growth gradually ceases and tissues ultimately die. No growth of adult tissues could be obtained in such a fluid, and it was noticed that although embryonic tissues grew well, tumours were far more difficult to grow in such a fluid than in plasma. The addition of glucose to the fluid gave no better results. As such fluids contain no nitrogen the addition of rat serum was tried, but the resulting growths were no greater than were obtained without it. The addition of embryo extract was also tested. The extract was prepared by finely mincing mouse or rat embryos in a small quantity of the above saline solution, and freezing and thawing the mass two or three times so as to disintegrate the cells as much as possible, and then



centrifuging till a clear or faintly opalescent fluid was obtained. Cultures were put up containing 2 parts of such an extract with 3 of the saline solution, and very rapid and vigorous growths were obtained both of embryonic tissues and tumours. These cultures could be subcultured apparently indefinitely, and increased greatly in bulk. Growths of adult tissues could also be obtained, though with far greater difficulty. Once growth started, however, it was easy to keep it growing in subculture.

It seems evident from these experiments that for the continued growth of tissues outside the body at least two factors are necessary—a saline solution in which the calcium salts are probably in the colloidal state, and some substance or substances contained in an extract of embryonic cells. Indeed Carrel and Ebeling (1921), in a recent paper, appear to consider that the indefinite multiplication of cells in a medium is due entirely to substances contained in embryonic juice. This conclusion is supported, not only by the above experiments, but by the fact that continuous growth cannot be obtained in plain plasma cultures unless embryonic extracts are added to them. Heat at 60° C. for an hour seems to impair greatly the efficacy of the extracts. Whatever the substance or substances in embryo extracts are, it appears unlikely that they are merely a product of all rapidly-growing tissues, as tumour extracts cannot replace embryo extract. It was further found that proliferating connective tissue furnished an extract which was inert. This tissue was obtained by inoculating diatomaceous earth subcutaneously into rats. The extraction was made in the manner already described for making embryo extract. The plasma of a pregnant animal was tried, but it also could not replace embryo extract, so that, whatever the substances are, they are evidently not present in the maternal circulation. As has already been stated, a certain limited amount of growth can be got from both tumours and embryonic tissues in a plain plasma medium. It seems probable, therefore, that in such cases where limited growth occurs without the addition of extract, it must be due to substances carried over with the original fragment. When this is used up growth ceases. Tumours would, on this view, contain some of the necessary substances for growth, but evidently not in anything like the amount that embryonic extracts do, and, judging from these experiments, the adult tissues of the mouse and rat must contain little or none. A point arises in this connection of great interest, viz. that although adult mouse tissues are most difficult to cultivate, yet tumours will grow comparatively easily, and show in most cases abundant growth of the stroma as well as of parenchyma. The stroma growth is, of course, derived from the host, and it therefore appears that the tumour has a very definite action on the connective tissues of its host, enabling them to grow in a similar manner to embryonic tissues outside the body.

Complete loss of differentiation does not appear to be a necessary consequence of successful culture *in vitro*, especially in fluid media. Thus, heart may be grown for generation after generation, all traces of the original fragment having disappeared, and the growth consisting of a thin, rapidly growing, pulsating sheet of cells. These cells if suitably fixed and stained (alcohol acetic or Fleming fixation, followed by iron-haematoxylin) show typical myofibrillæ. Most of the American observers have described heart

growths as consisting of fibroblasts. Lake (1916), who observed pulsation of individual cells, was the first to suggest that these were really muscle-cells. There can be no doubt that this is the correct view, the cells in most cases being typically striated as shown in Plate II, fig. 3*a*, and Plate III, fig. 3*b*. It has been stated that no pulsation occurs in heart cultures till a certain amount of tension has been obtained by contraction of the medium. Cultures in this liquid medium, however, frequently show active pulsation when the fragment is floating free, so that external tension is out of the question.

Cultures of embryo skin present interesting features. Growth spreads out from the original fragment as a thin sheet of shorter or longer spindle-cells showing active mitosis (Plate IV, fig. 4), and growth continues in this form

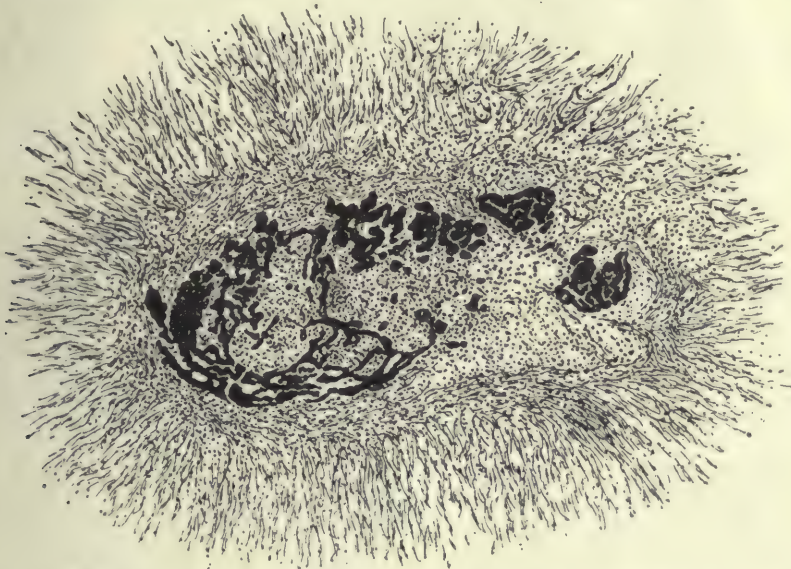


FIG. 7.—Five-day culture of mouse carcinoma 206, showing acinous type of growth of parenchyma with abundant stroma growth.  $\times 50$ .

if sub-cultured every three or six days. If, however, the culture be left to itself for five or six days, keratinisation begins (Plate IV, fig. 5). Cultures of mammary carcinoma such as strain 206 of this laboratory show at first only a rapid growth of stroma. In forty-eight hours, however, the parenchyma commences to push through the stroma in the form of finger-shaped processes (Plate V, fig. 6). These gradually spread out and in later subcultures show an acinous appearance (Text-fig. 7). In some cases the stroma tends to die out after the first subculture and a pure growth of parenchyma is obtained.

All such cultures show that there is a peculiar relationship between the fibroblasts and the epithelial cells. For example, in cultures of carcinoma 206, where an abundant growth of stroma as well as of parenchyma is obtained, the parenchyma shows an acinous type of structure in the later subcultures as growth progresses. If, however, this tumour be subcultured very frequently,



from the first the growth of parenchyma is free from stroma. Such a growth merely forms a mass of undifferentiated cells with no tendency to form acini. Similarly while skin is growing with a large amount of connective tissue keratinisation is frequent, but when, as occasionally happens, the skin grows out as a sheet away from the connective-tissue cells, no keratinisation occurs and growth continues as a mass of rapidly dividing spindle-shaped cells. The same thing applies to heart. Where the heart-cells grow in proximity to connective-tissue cells they show typical myofibrillæ, but if the connective-tissue growth fails, or the heart-cells grow away from the connective tissue, they appear merely as spindle-shaped cells and myofibrils are absent, though the beat does not seem to be affected by such a change, at all events for some generations. Tissues growing badly, either through the medium being not quite suitable or through insufficient subculture, commence to show degenerative changes. This in its earliest form shows itself as an accumulation of granules within the cytoplasm till the cell becomes packed. These granules give all the reactions of fat, and if cells be examined in the early stages of degeneration, the fatty granules may be seen to arise from a transformation of the mitochondria, in the same way as has been described by many authors in normal and pathological fat formation.

#### DISCUSSION.

The continued growth of embryonic tissues is easily obtained outside the body in suitable coagulated or liquid media. It is notorious that when transplanted into living animals continued growth does not occur. At the most, survival of cells after temporary multiplication is obtained. The cells of malignant new growths present, at first sight, a remarkable contrast. Continued growth is easily obtained in the living animal, but *in vitro* early degeneration and death necessitate frequent subculture if even a limited growth is to be achieved. These experiments show that this early death of the cells is due to elaboration of toxic substances, which quickly reach a lethal concentration. In addition to a proper colloidal condition of the calcium salts, which is essential in the fluid medium replacing the complicated coagulated plasma, previously used almost exclusively for this kind of work, a substance or substances not heretofore found except in extracts of embryonic tissues is necessary for continued growth of all cells *in vitro*. It has not been demonstrated in rapidly growing tumours nor in regenerating tissues of the adult so far as our experience has gone. It is not possible at present to say whether this growth-promoting substance plays a part in the growth of normal adult tissues (*e.g.* in regeneration) and of tumours *in vivo*, or whether its importance in growth *in vitro* is mainly a consequence of the technical environmental conditions involved.

The experiments bring out very clearly the close biological relations subsisting between tumour parenchyma and stroma. The stroma of a transplanted tumour, although derived from the adult host in which the tumour is growing for the time being, behaves in culture like an embryonic tissue rather than like that of an adult. The presence of stroma elements, although not necessary for the growth of the parenchyma *in vitro*, exerts a profound

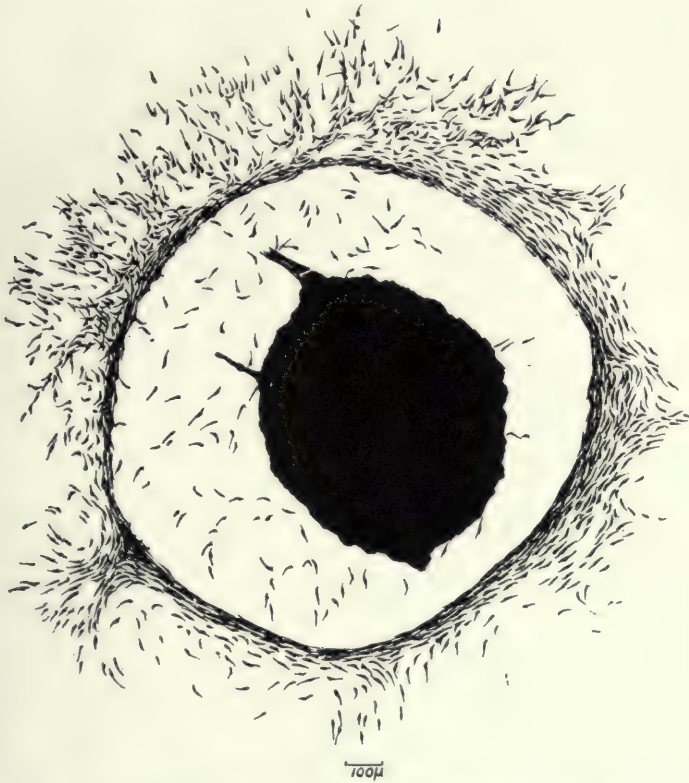


FIG. 2 *a*.—Plasma culture of 37 S. sarcoma, 24 hours' growth, showing liquefaction of plasma round original fragment and typical ring-shaped growth of the sarcoma cells.

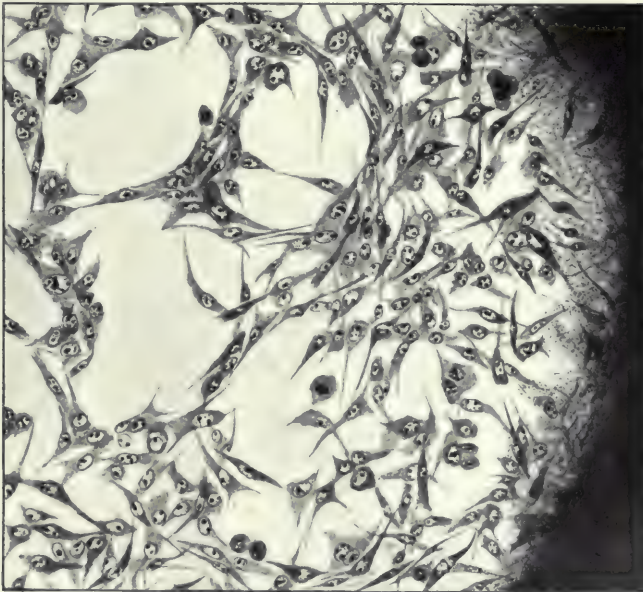


FIG. 2 *b*.—Portion of sarcoma culture in fig. 2 *a*, showing spindle cells.  $\times 250$ .





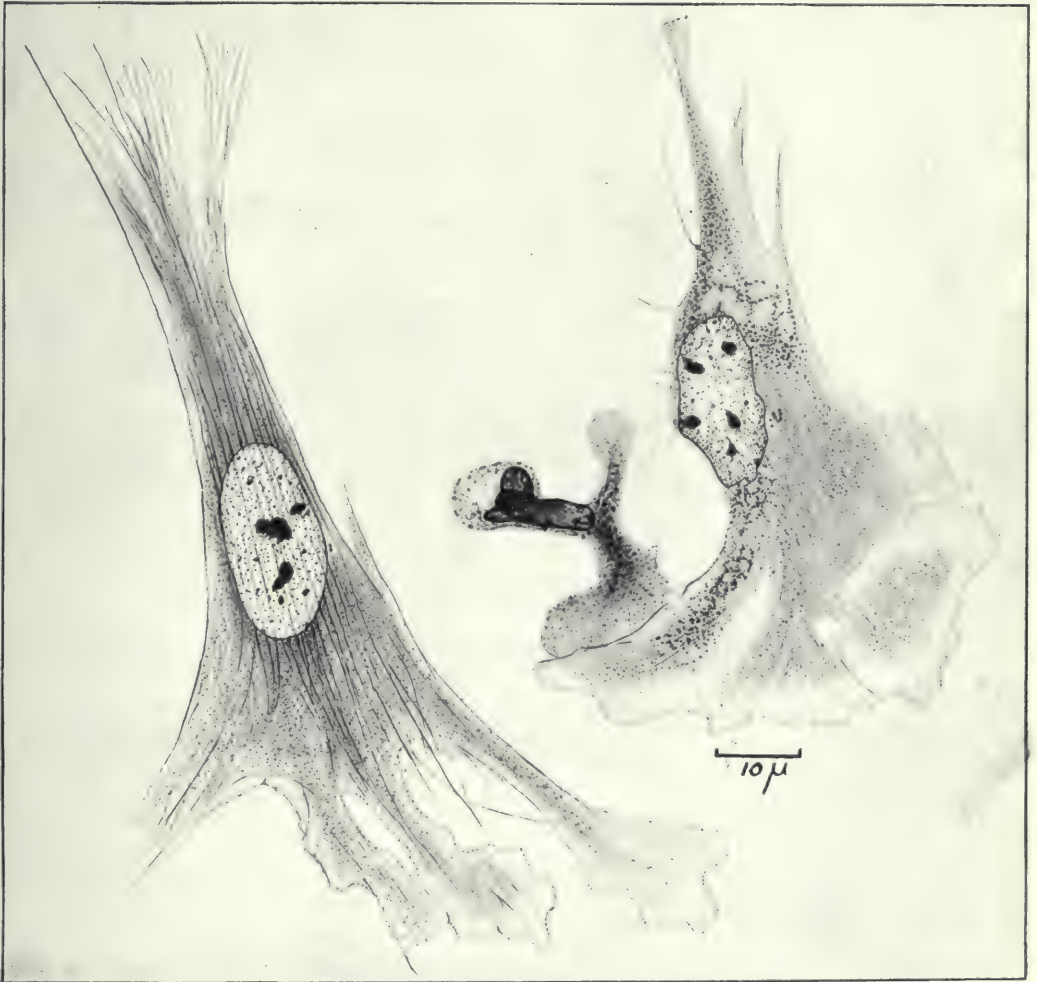


FIG. 3 *a*.—Isolated cells from culture of embryo mouse heart in saline medium, showing myofibrillæ.





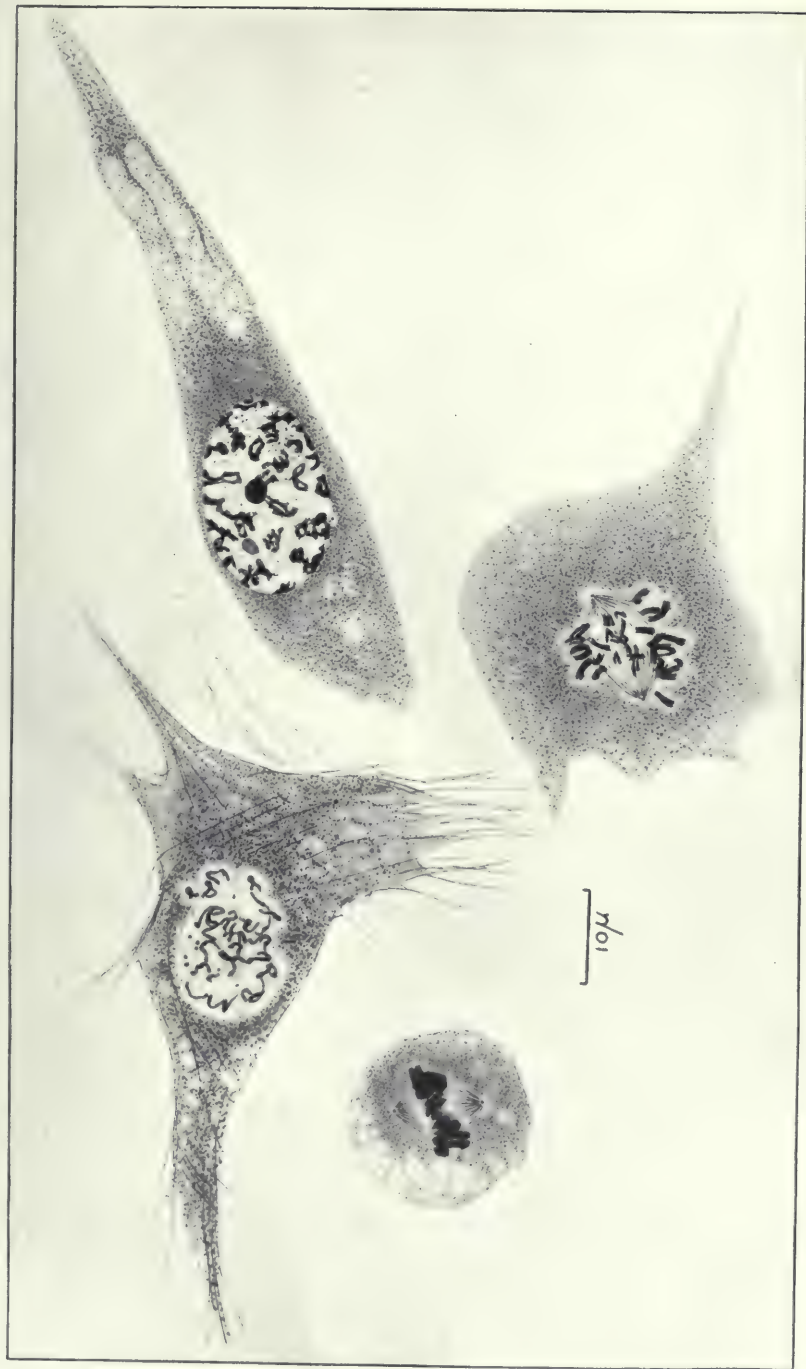


FIG. 3 *b*. — Isolated cells from culture of embryo mouse heart in saline medium, showing mitotic figures.





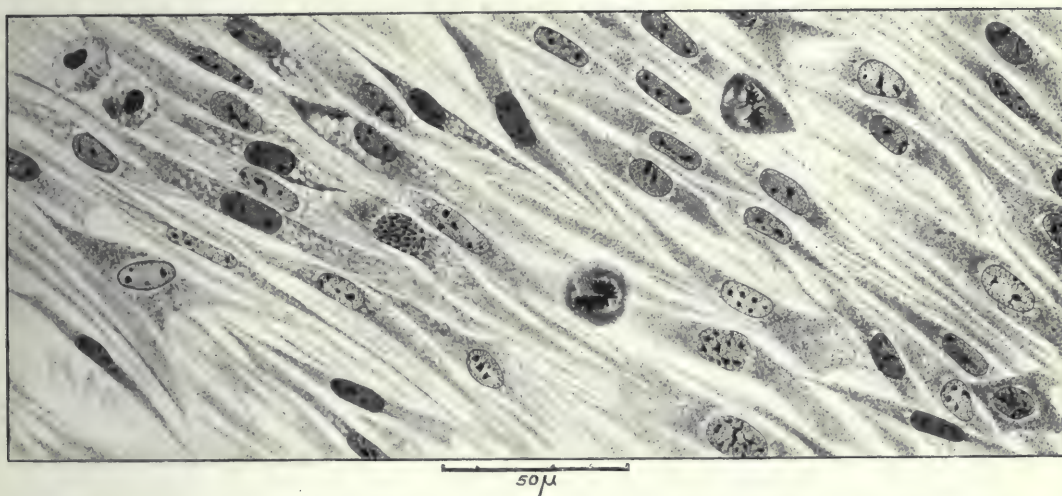


FIG. 4.—Young culture of embryo mouse skin in plasma medium, showing spindle-celled type of growth.

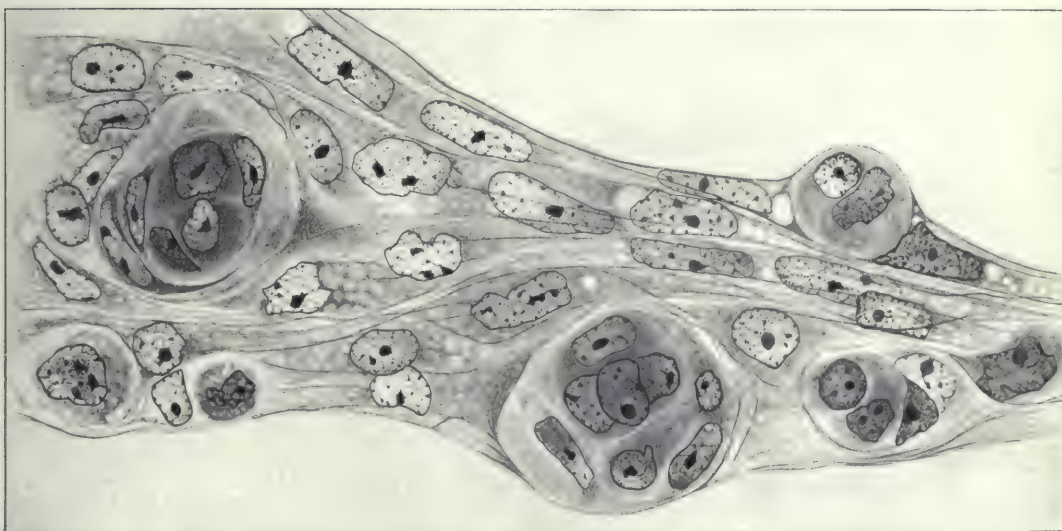


FIG. 5.—Plasma culture of embryo mouse skin, showing commencing keratinization.  $\times 1500$ .





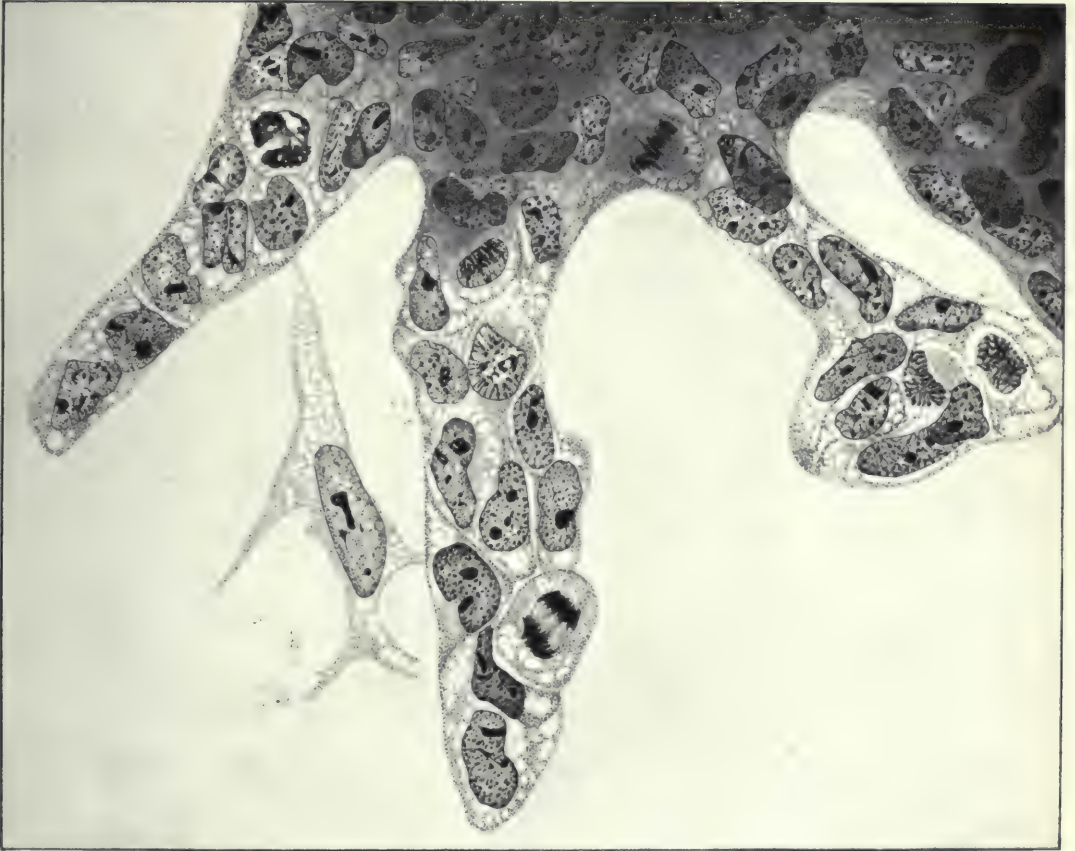


FIG. 6.—Young culture of mouse carcinoma 206, showing growth as finger-shaped processes.  
× 1500.





influence upon it, and in particular partly determines the degree of differentiation reached by the tumour-cells. The analysis of the factors responsible for these relations of cells to their environment is being made the subject of continued study.

#### SUMMARY.

Continued growth of normal and tumour-cells *in vitro* is only obtained in the presence of substances so far only found in embryonic extracts. A salt mixture in which the calcium is present in the colloidal state can, with the addition of embryo extract, replace plasma for this work. The degree of differentiation shown by cultures of normal and malignant tissues is partly conditioned by the accompanying growth of the stroma. The stroma behaves in culture like embryonic tissue rather than like the adult tissues from which it is derived.

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[All the figures have been drawn by Mr. W. Pilgrim, of the laboratory staff.]



## ON UREA TESTS OF RENAL FUNCTION.

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IN the course of some combined clinical and laboratory work on diabetes mellitus and certain glycosurias\* I am attempting to gauge the condition of the kidneys by simple renal function tests. For this purpose the urea tests, among others, are being used. Partly as a result of these experiments and partly from the experience gained from the routine examination of hospital patients with kidney disease several facts of importance have come to light. It is felt that these points may be of interest to those more directly studying renal efficiency. In estimating the concentration of urea in the urine (hypobromite method) and in the blood (urease method) the techniques outlined by Maclean and de Wesselow (1919, 1920) were followed. Blood-urea estimations were always performed in duplicate and the mean taken.

### THE PROVOCATIVE UREA CONCENTRATION TEST.

For details of this test the reader is referred to the publications of MacLean and de Wesselow (1919, 1920). In the great majority of 400 cases the results have been most helpful. But occasionally an interpretation of the figures has been difficult or misleading clinically, and for this reason the following questions have been raised:

#### A. *For how long before the Administration of the Urea should Fluids be withheld?*

I believe it is the custom of MacLean and his co-workers to stop all fluids for 6–12 hours before the test, but I would suggest that the necessity for this precaution has not been given sufficient emphasis in all their publications. For instance, Walker (1921), investigating urea concentration tests in the psychoses, allowed tea  $2\frac{1}{2}$  hours before the dose of urea. Yet he states that he carefully followed the prescribed details (MacLean and Russell, 1920). It is clear that discrepancies may arise if the test is not carried out under standard conditions. Most healthy active individuals would seem able to concentrate to well over 2 per cent. in the second hour in spite of taking fluids within half an hour to three hours before the urea. Thus H— imbibed  $2\frac{1}{4}$  pints of fluid  $1\frac{1}{2}$  hours before the test, and yet his urea percentage was 2·30 at the end of the second hour (Table I, No. 107). On the other hand, patients with

\* On behalf of the Medical Research Council.

renal disease may yield very varying concentrations if allowed different quantities of fluid at short intervals before the test.

TABLE I.—*The Effect of taking Fluid shortly before the Test.*

No.	Case.	Quantity of fluid and interval before test.	Urea per cent. and c.c. of urine at end of—			Remarks.
			1st hour.	2nd hour.	3rd hour.	
90	H—	1½ pints; 1 hour	2·80; 62	2·64; 90	3·10; 63	Healthy active individual ( <i>cf.</i> No. 157).
91	"	1½ " 1 "	1·12; 265	2·34; 107	2·85; 82	
107	"	2½ " 1½ hours	0·67; 325	2·30; 94	2·29; 86	
113	A—	½ pint; ½ hour	0·59; 230	1·46; 120	2·18; 60	Pyelonephritis.
116	"	¼ " 3 hours	2·32; 78	2·68; 64	2·36; 49	
132	W—	1 " 2 "	0·96; 240	1·63; 120	1·52; 120	Chronic nephritis (mixed).
156	"	<i>Nil</i> 12 "	2·67; 90	3·03; 45		
33	G. H—	1 pint; 1 hour	0·93; 530	1·45; 130		"Debility." No evidence of renal disease. History of trench nephritis ( <i>cf.</i> No. 107). Enlarged prostate.
42	"	<i>Nil</i> 12 hours	2·16; 50	3·69; 50		
157	T—	½ pint; 3½ "	0·91; 330	2·60; 60		
177	S—	1 " ½ hour	1·49; 360	1·82; 210		Congenital syphilis.
178	"	<i>Nil</i> 12 hours	2·08; 120	2·04; 90		
222	McL—	1 pint; 2 "	2·02; 240	2·20; 240	1·83; 60	
225	"	<i>Nil</i> 12 "	2·52; 105	2·50; 90	2·56; 90	
46	N—	1½ pints; 1 hour	0·72; 750	1·65; 120		"Lumbago."
60	"	1 pint; 2 hours	0·96; 235	1·62; 125		
191	"	<i>Nil</i> 12 "	2·13; 90	2·46; 66		

I assume that it is desirable to stimulate the kidneys to concentrate to their utmost under the conditions of the test. Two rules should therefore be observed:

(1) Withhold all fluids for at least 6 hours before the dose of urea.

(2) Measure separately the volume of urine passed in each hour subsequently. If the concentrations are well above 2 per cent. the volumes do not matter, but if in the first hour more than 150 c.c. of urine are excreted, or in the second or subsequent hours more than 100 c.c., with a urea concentration below 2 per cent., I would suggest that diuresis may be the cause of the low results and would repeat the test, controlling it by other methods, *e.g.* blood-urea estimation. Even though all fluids are withheld for 6–12 hours beforehand, a few patients may yield artificially low results if they have been taking large quantities of fluid: presumably water has been retained and the urea has enabled the kidneys to eliminate some of it (Table II, Case C—). In diabetes mellitus such polyuria may be troublesome (Table II, Case C. J—). In a few patients with glycosuria I have obtained normal results after their urine has become free from sugar, whereas before treatment the concentration was below 2 per cent. (Table II, Case M—). In the majority of the cases of diabetes, however, the urea concentration has been normal before treatment, often in spite of polyuria (Table II). The most obstinate example of diuresis with which I have met occurred in a patient with diabetes insipidus (Table II, Case D—). Two experiments yielded extremely low results, yet the blood urea



was only 34 mgrm. per 100 c.c. After 1 c.c. pituitrin subcutaneously the concentration at the end of the second hour was 2.94 per cent.

TABLE II.—*Diuresis not Controlled by Withholding Fluids 8 to 12 Hours before Test.*

No.	Case.	Diagnosis.	Urea per cent. and c.c. of urine at end of—			Remarks.
			1st hour.	2nd hour.	3rd hour.	
379	C—	Unilateral malignant disease of kidney	2.66; 45	2.41; 75	2.36; 60	On ordinary amount of fluids beforehand.
416	„		1.68; 480	1.94; 120	2.00; 120	On large quantities of fluid for several days beforehand.
255	C, J—	Diabetes mellitus	1.59; 230	1.84; 188	2.34; 122	Marked glycosuria.
140	M—	Cirrhosis of liver and glycosuria	1.36; 176	1.87; 130		Marked glycosuria.
267	„			2.32; 178		1st and 2nd hours combined. No glycosuria.
265	R—	Diabetes mellitus	2.32; 150	2.45; 120	2.46; 120	Considerable glycosuria.
275	„		1.84; 90	2.47; 60	2.59; 90	Test performed after treatment. No glycosuria.
51	D—	Diabetes insipidus	0.74; 235	0.66; 330		Blood urea, 34 mgrm. per 100 c.c.
65	„		1.15; 142	0.79; 250		
114	„		2.41; 120	2.94; 120		After 1 c.c. pituitrin subcutaneously.
426	K—	Diabetes mellitus	0.93; 270	2.20; 90	2.26; 60	Test performed after treatment. No glycosuria.
131	H—	Diabetes mellitus	1.35; 166	2.14; 149	2.60; 62	Very slight glycosuria at time of test.

#### B. Is 15 grm. a Sufficient Dose of Urea in all Cases?

So much urea may be excreted in the first hour or in the first 2 hours (*e. g.* Table I, No. 222) that there is no longer a sufficient "head" to provoke a concentration which is the maximum for the individual. This may happen even when fluids have been withheld for 8 hours or more (Table II, Case C). Of course where no care has been taken to control diuresis, the resultant polyuria is usually the cause of low results (Table I, Case N).

Secondly, it is conceivable that occasionally the rate of absorption of urea from the alimentary tract may be so delayed that the kidneys have not the opportunity to yield their maximum concentration. Seven cases of gastrointestinal disease have, however, given normal results (Table III). One case of diabetes and one of chronic parenchymatous nephritis show quite definitely the effect of increasing the dose of urea (Table IV), but it would not be fair in the absence of blood-urea estimations to offer these as evidence of poor absorption. I have not made any experiments with urea given intravenously. The absorption of urea appears to be increased slightly by taking food (*e. g.* breakfast—no fluids) just before the test (Table V).

TABLE III.—*Effect of Gastric and Intestinal Diseases.*

No.	Diagnosis.	Urea per cent. and c.c. of urine at end of—		Remarks.
		1st hour.	2nd hour.	
53	Chronic gastritis	2.45; 120	2.53; 90	Mild attack. Confirmed at autopsy.
101	Appendicitis	4.52; 60	4.65; 90	
169	Carcinoma of stomach	2.09; 90	2.18; 75	
195	Carcinoma of transverse colon	3.06; 30	3.09; 60	Confirmed at laparotomy.
312	Duodenal ulcer	1.52; 180	2.75; 85	Confirmed at operation.
57	Cirrhosis of liver and gastritis	3.54; 15	4.02; 45	
62	Cirrhosis of liver and gastritis	2.65; ?	3.20; ?	

TABLE IV.—*Effect of Increasing the Dose of Urea.*

No.	Diagnosis.	Urea per cent. and c.c. of urine at end of—				Remarks.
		1st hour.	2nd hour.	3rd hour.	4th hour.	
167	Chronic parenchym. nephritis	1.40; 25	1.52; 35			10 grm. of urea. Age 8.
172	Diabetes mellitus	1.96; 30	2.12; 30			15 " " "
385		1.05; 210	1.54; 60	1.68; 90		15 " " " Age 30.
390		0.83; 180	1.29; 98	1.57; 90		15 " " "
396		1.31; 240	1.98; 120	2.38; 120	2.11; 120	30 " " " There was no evidence, clinical or otherwise, of renal disease in this patient.

TABLE V.—*Effect of Food (no Fluids) on the Absorption of Urea.*

No.	Diagnosis.	Urea per cent. and c.c. of urine at end of—			Remarks.
		1st hour.	2nd hour.	3rd hour.	
426	Diabetes mellitus	0.93; 270	2.20; 90	2.26; 60	No fluid and no food for 12 hours.
427		1.05; 120	2.03; 120	2.43; 30	No fluid for 12 hours but breakfast one hour before test.
454	Bilateral congenital cystic disease of kidneys	1.53; 30	1.46; 60	1.45; 90	No fluid and no food for 8 hours.
459		2.16; 60	2.24; 30	2.06; 90	No fluid for 8 hours but breakfast (bread and butter) 2 hours before test.
460	Tuberculosis of kidney (unilateral)	1.25; 60	1.53; 60	1.70; 60	No fluid and no food for 8 hours.
461		1.79; 96	1.91; 88	2.05; 64	No fluid for 8 hours but breakfast (bread and butter and 1 egg) 2 hours before test.



*c. To what Extent will Proteinuria Introduce Errors ?*

It has long been known that sodium hypobromite reacts with "albumen," liberating nitrogen. On inquiring I found that MacLean and de Wesselow allowed for this by including a certain number of albuminous urines when working out their factor for converting the volume of nitrogen evolved into urea per cent. In the vast majority of instances, from a clinical standpoint, I find that proteinuria makes no appreciable difference. The interpretation of results in the region of 2 per cent., however, may be troublesome. Very occasionally the error is considerable (Table VI). In practice, except when the result is close to 2 per cent., I have found it unnecessary to remove protein unless it exceeds 0.4 per cent. (Aufrecht). Sometimes concentrations, even

TABLE VI.—*Error Introduced by Proteinuria.*

Protein per cent. (Aufrecht).	Influence of removal of protein on urea per cent.			Remarks.	Disease.	No.
	Before removal.	After removal.	Differ- ence.			
0.03	2.05	1.84	0.21	Removal of protein brings result below 2 per cent.	Renal calculus	12
0.09	2.05	2.05	0.00	Protein makes no difference	Trench nephritis	5
0.14	2.33	2.32	0.01	Protein makes no difference	Cirrhosis of liver	267
0.18	2.05	1.87	0.18	Removal of protein brings result below 2 per cent.	Chronic interstitial nephritis	436
0.24	2.61	2.39	0.22	Protein makes little difference to interpretation	Subacute nephritis	133
0.21	2.31	2.26	0.05	Protein makes no difference	Malignant growth of bladder	440
0.35	2.84	2.02	0.82	Protein introduces large error	Malarial nephritis	6
0.39	2.21	1.96	0.25	Removal of protein brings result below 2 per cent.	Albuminuria of pregnancy	442
0.42	1.90	1.66	0.24	Protein makes appreciable difference	Chronic parenchymatous nephritis	31
0.51	0.69	0.63	0.06	Relatively large amount of protein does not matter	Chronic nephritis (mixed)	142
0.66	1.25	1.20	0.05	Large amount of protein makes no difference	Chronic nephritis (mixed)	450
0.75	2.20	1.52	0.68	Proteinuria introduces grave error	Chronic nephritis (mixed)	9

*Method of removal of protein.*—To 40 c.c. of urine 10 c.c. of 25 per cent. salicyl-sulphonic acid are added. After mixing and filtration, a small portion of the filtrate is tested by further addition of the reagent, to confirm freedom from protein. The urea is estimated in 4 c.c. of the filtrate in the usual way, and the volume of nitrogen evolved multiplied by 5/4. Or, more simply, 5 c.c. of filtrate, which is equivalent to 4 c.c. of untreated urine, may be utilised, the calculation being then as for 4 c.c. of urine.

exceeding this figure, introduce very little error; the urinary proteins of different patients would seem to vary in the readiness with which they react with hypobromite to yield nitrogen. It is interesting to note that urines deeply reddened by blood may not contain as much as 0.4 per cent. (Aufrecht) protein. One of the great advantages of the urea concentration test is that ordinary degrees of hæmaturia do not matter.

#### ESTIMATION OF CONCENTRATION OF UREA IN BLOOD.

Since urea is very largely exogenous in origin, its concentration in the blood should vary with the nature of the diet. Such has been found to be the case by many observers (MacLean, 1921), and is shown very markedly by estimating the blood urea with and without a dose of urea by mouth (Table IX).

Quite frequently patients with kidney disease are allowed little or no protein. On such diets the blood urea result obtained before an operation (*e. g.* prostatectomy) may sometimes be dangerously reassuring. Furthermore, estimations made on different occasions upon the same patient may not be comparable owing to differences in the diet.

#### UREA CONCENTRATION FACTOR (U.C.F.).

Unfortunately it is not possible to work out blood-urea curves on similar lines to blood-sugar curves because a satisfactory microchemical method for the estimations is not available. But the conditions would be more standardised if the measurements were made at a known time after a known dose of urea (by mouth). For this purpose it has been my custom for several months to combine the urea concentration test (urine) and the blood-urea estimation. In the majority of cases, after a dose of 15 gm. (by mouth) the percentage in the urine reaches a maximum at the end of the second hour, and stays at about the same level during the third hour. Therefore I have selected the middle of the third hour for estimating the blood urea, and have termed the quotient,

$$\frac{\text{mgrm. urea per 100 c.c. urine passed during the third hour}}{\text{mgrm. urea per 100 c.c. blood in middle of third hour}},$$

the "urea concentrating power." But MacLean (1921) has already published the principle of dividing the urinary urea by the blood urea, although, to my knowledge, he has not definitely advocated the simple expedient of combining the two tests, and thus simultaneously checking the urea concentration (urine) and standardising the blood urea. I shall therefore adopt the name he suggests, and refer to the combined test as the "urea concentration factor," or, for short, U.C.F. There is always a possible danger in confining investigations to one side of the kidney. The U.C.F. avoids this. The following example illustrates the point well (Table VII).

The patient suffered from chronic nephritis with both chloride and urea retention. His urea concentration (urine) during the third hour after 15 gm. of urea was 1.14 per cent. (volume of urine 90 c.c.) on April 29th, 1921. A month later it was 1.50 per cent. (180 c.c.), and a week after that 1.72 per cent. (60 c.c.). In short, judging by these results alone, the condition of his kidneys would seem to have improved. But the corresponding blood-urea results in



the middle of the third hour were 62, 180 and 240 mgrm. per 100 c.c. respectively, the resulting urea concentration factors being 18, 8 and 7, thus clearly suggesting that the renal disease was worse. Furthermore, a month after the third test the urea concentration had fallen to 1.37 per cent. (90 c.c.), and after a further three and a half months it was 1.33 per cent. (54 c.c.), but contrary to what might have been expected this probably implied an improvement, because the blood urea had fallen to 136 mgrm. and 113 mgrm. (middle of third hour) respectively, with resultant U.C.F.'s of 10 and 12.

TABLE VII.—*Case Illustrating the Importance of Combined Tests (U.C.F.).*

No.	Date.	Urea per cent. and c.c. of urine at end of—			Blood urea (mgrm. per 100 c.c.). Middle of third hour.	U.C.F.	Remarks.
		1st hour.	2nd hour.	3rd hour.			
333	29/4/21	1.24; 50	1.18; 70	1.14; 90	62	18	{ Urea concentration (urine) rising, but blood urea also rising; U.C.F. falls; kidney lesion probably becoming worse.
351	27/5/21			1.50; 180	180	8	
357	3/6/21	1.83; 120	1.79; 120	1.72; 60	240	7	
363	29/6/21	1.28; 90	1.36; 90	1.37; 90	136	10	{ Urea concentration (urine) falling, but blood urea also falling; U.C.F. rises; kidney lesion probably improving.
450	13/10/21	1.20; 75	1.28; 70	1.33; 54	113	12	

(For further examples see Table IX, *e. g.* Nos. 461 and 462.)

The obvious criticism of the U.C.F. as thus defined is (1) that the urinary urea is estimated by the hypobromite method instead of by the more accurate urease method, and (2) that the selection of the middle of the third hour for estimating the blood urea is arbitrary. The answer is that the test is suggested as being of greater clinical value than either the urea concentration (urine) alone, or the blood urea without control of diet, or the results of the two tests performed separately; it obviously must yield only an approximation, but in the present state of knowledge of the rate of absorption of urea in different pathological conditions it probably yields a result sufficiently accurate for clinical interpretation; it would only increase the time required for the test to estimate the urinary urea by the urease method, and so in practice would limit its application. Whenever the blood-urea has to be estimated it gives very little extra trouble to arrange to take the sample in the middle of the third hour.

#### THE UREA COEFFICIENT (AMBARD).

This coefficient has been shown by MacLean and de Wesselow (1920, 1921) to give no more information of clinical value than an estimation of blood-urea.

## DISCUSSION OF RESULTS.

MacLean and de Wesselow regard the urea concentration (urine) as normal if between 2 and 4 per cent. at the end of the second hour. Moreover, they say, ". . . if below 2, the condition is unsatisfactory, and the lower the concentration the more serious the lesion" (1920). An example has already been given (Table VII, Nos. 357, 363 and 450) showing that the second half of the sentence quoted is not always strictly true, for occasionally the urea concentration (urine) may fall because the blood-urea has also fallen, the lesion presumably having improved. At the present time I would interpret the results of this test as follows: If the urea concentration (urine) at the end of the first, second or third hour exceeds 2·5 per cent. it is "normal," *i. e.* probably more than one-quarter of the total kidney tissue was functioning on the day of the test; probably little or no further information of value clinically would be obtained by estimating the blood urea. If the concentration lies between 2 and 2·5 per cent. it is probably "normal," but it would be safer to check it by estimating the blood urea (preferably 2½ hours after the dose of urea). If the urea concentration is below 2 per cent. it may be due to an artefact, or the renal condition may be definitely unsatisfactory, less than one-quarter of the kidney functioning. As examples of artefacts there may have been diuresis, the full dose may not have been taken, or the dose may even have been omitted, the solution may not have contained 15 *grammes* (sometimes 15 *grains* have been prescribed in error!), and so on; if there is any reason to suppose that the test has not been carried out correctly it should be repeated. An estimation of urea in the 24 hours' urine may serve as a useful check; if it is higher than the second- or third-hour samples the test has not been

TABLE VIII.—*Urea in Urine above 2 per cent., and Blood Urea above 40 mgrm.*

No.	Disease.	Urea per cent. and c.c. of urine at end of—			Blood urea. (No urea by mouth.) (Mgrm.)	Remarks.
		1st hour.	2nd hour.	3rd hour.		
153	Subacute nephritis	1·61; 70	2·08; 90	2·24; 70	49	Recovery.
388	Vesical calculus	2·16; 90	2·32; 90	2·41; 60	73	Successfully removed by operation.
405	Enlarged prostate	1·84; 30	2·26; 30	2·13; 15	44	Successful prostatec- tomy.
379	Malignant tumour of kidney (uni- lateral)	2·66; 45	2·41; 75	2·36; 60	92	Successful nephrec- tomy.
436	Chronic inter- stitial nephritis	1·87; 90	2·06; 60	1·90; 60	85	Blood-pressure(systolic) 260. Marked albumin- uric retinitis.
447	Chronic nephritis	1·96; 90	2·00; 120	1·99; 60	76	Marked dyspnœa. Blood-pressure 190/120. Fundi normal. Was- sermann reaction posi- tive.



TABLE IX.—Results Obtained by Combined

## GROUP 1.—Urea Concentration

No.	Disease.	Date	Urea per cent. and c.c. of urine at end of—		
			1st hour.	2nd hour.	3rd hour.
370	Talipes equino-varus . . . . .	8/7/21		3.80; 90	4.73; 60
378	Chronic nephritis (mixed) . . . . .	12/7/21	2.98; 36	3.16; 32	
400	Calculus in right kidney; tuberculosis of left kidney . . . . .	29/7/21	2.03; 30	2.42; 60	2.19; 30
432	Diabetes mellitus . . . . .	19/9/21	3.09; 100	3.11; 90	3.10; 85
360	Liporhabdomyoma of left kidney . . . . .	6/6/21	4.00; 120	4.04; 60	3.99; 60
367	L. pyonephrosis . . . . .	4/7/21	2.11; 120	2.08; 30	2.09; 30
453	Postural proteinuria (? functional) . . . . .	15/10/21	2.33; 70	2.48; 70	2.70; 70
426	Diabetes mellitus . . . . .	13/9/21	0.93; 270	2.20; 90	2.26; 60
430	Diabetes mellitus . . . . .	16/9/21	2.42; 120	2.64; 120	2.57; 90
372	Malignant growth of bladder . . . . .	11/7/21	2.48; 45	2.81; 60	2.88; 90
405	Enlarged prostate . . . . .	30/7/21	1.84; 30	2.26; 30	2.13; 15
455	Carcinoma of stomach and chronic nephritis (interstitial) . . . . .	15/10/21	2.03; 75		2.18; 86
436	Chronic nephritis (interstitial) . . . . .	26/9/21	1.87; 90	2.06; 60	1.90; 60
388	Vesical calculus . . . . .	22/7/21	2.16; 90	2.32; 90	2.41; 60
379	Malignant tumour of kidney (unilateral) . . . . .	12/7/21	2.66; 45	2.41; 75	2.36; 60
447	Chronic nephritis . . . . .	8/10/21	1.96; 90	2.00; 120	1.99; 60

## GROUP 2.—Urea Concentration

399	Diabetes mellitus . . . . .	29/7/21			1.41; 180
337	Post-scarlatinal nephritis . . . . .	5/5/21	1.43; 60	1.62; 90	1.38; 60
393	Enlarged prostate . . . . .	25/7/21	1.77; 30	1.47; 60	1.83; 90
404	Enlarged prostate . . . . .	30/7/21	1.55; 30	0.93; 5+	1.32; 15+
374	Enlarged prostate . . . . .	11/7/21	1.04; 60	1.24; 90	1.75; 30
330	Chronic interstitial nephritis . . . . .	26/4/21	0.95; 90	1.17; 120	1.04; 60
444	Chronic interstitial nephritis . . . . .	30/9/21	1.38; 120	1.26; 120	1.39; 120
366	Malignant prostate disease . . . . .	2/7/21	0.55; 68	0.59; 30	0.69; 30
307	Chronic interstitial nephritis . . . . .	31/3/21	0.71; 90	0.78; 60	0.83; 60
371	Right renal calculus; left cystic kidney . . . . .	11/7/21	1.49; 60	1.02; 60	1.16; 90
448	Perineal fistula . . . . .	12/10/21	1.03; 45	1.21; 60	1.38; 45

## GROUP 3.—Example of identical U.C.F. for two cases,

461	Tuberculosis of left kidney . . . . .	21/10/21	1.79; 96	1.91; 88	2.05; 64
462	Renal calculus . . . . .	21/10/21	1.59; 68	1.68; 93	1.70; 74

Remarks on Nos. 461 and 462.—Judging by urea concentration test (urine) alone, 461 would seem judging by the U.C.F. the two

*Tests (Urea Concentration Factor).*

(urine) 2 per cent. or higher.

Blood urea 2½ hours after dose of urea. (Mgrm.)	U.C.F.	Date.	Blood urea. (No urea by mouth.) (Mgrm.)	Remarks.
66	72	9/7/21	27	No evidence whatever of renal disease. Successful cuneiform osteotomy of foot.
53*	60	...	...	*Blood urea two hours after dose of urea.
37	59	...	...	Successful right nephrolithotomy.
58	53	...	...	No evidence whatever of renal disease.
75	53	...	...	Successful nephrectomy.
42	50	...	...	Successful nephrotomy.
55	49	...	...	Wassermann reaction, weak positive.
53	43	...	...	No evidence whatever of renal disease.
63	41	...	...	No evidence whatever of renal disease.
70	41	13/7/21	34	Growth partially excised.
59	36	2/8/21	44	Successful prostatectomy.
78	28	...	...	Inoperable carcinoma.
92	21	23/9/21	85	Blood-pressure 260. Marked albuminuric retinitis.
131	18	25/7/21	73	Successfully recovered by operation.
133	18	14/7/21	92	Successful nephrectomy.
134	15	14/10/21	76	Marked dyspnoea. Blood-pressure 190/120. Fundi normal. Wassermann reaction positive.

(urine) below 2 per cent.

34	41	...	...	Pyuria. Pyæmia. Death from septic pneumonia. Low urea in urine may be due to artefact.
45	31	...	...	Slight hæmaturia.
91	20	...	...	Successful prostatectomy.
73	18	...	...	Survived suprapubic cystotomy. Died from uræmia within thirty-six hours of prostatectomy.
104	17	...	...	Successful subrapubic cystotomy. Died twelve days after prostatectomy from exhaustion. Clinically not uræmic.
66	16	...	...	Systolic blood-pressure 190. Albuminuric retinitis.
97	14	...	...	Systolic blood-pressure 160. Fundi normal.
85	8	...	...	Inoperable. Discharged from hospital.
105	8	19/3/21	67	Systolic blood-pressure 240.
140	8	...	...	General condition good. No clinical evidence of uræmia. Operation considered inadvisable.
165	8	...	...	Uræmic at time of test. Died few days later. Fractured pelvis 2/9/21. Ruptured urethra.

(1) belonging to Group 1, (2) belonging to Group 2.

59	35	...	Same patient as No. 400, three months later.
48	35		

to be the less severe; judging by the blood urea alone, 461 would appear the more serious; cases are about equally severe.



properly conducted. In the absence of any artefact, results below 2 per cent. should always be controlled by estimating the blood-urea (preferably  $2\frac{1}{2}$  hours after the dose), for they may be accompanied, for example, by a practically normal blood urea (Table IX, No. 337) or by a very high one (Table VII, No. 357), the first being a much less severe case than the second.

The normal figures for blood-urea on ordinary hospital diets are usually given as 15–40 mgrm. per 100 c.c. (MacLean, 1921), and it is generally considered that if the urea concentration (urine) is above 2 per cent. the blood-urea will be below 40 mgrm. But I have encountered a few cases with urea in urine above 2 per cent. and blood-urea above 40 mgrm. (Table VIII).

Walker (1921) has reported similar findings. Some writers have suggested that factors other than the diet or the kidneys may influence the level of the blood urea—for example, the toxæmia of acute febrile diseases (Wagner, 1920) or of acute intestinal obstruction (Louria, 1921). If this be so, it is still more important to estimate simultaneously the urea concentration in both blood and urine in assessing renal efficiency.

I have not examined a sufficient number of active healthy individuals to suggest what may be the “normal” blood-urea level  $2\frac{1}{2}$  hours after a dose of 15 gm. by mouth. In 40 hospital patients, however, with a urea concentration (urine) over 2 per cent., the lowest has been 37 mgrm. and the highest 133 mgrm. per 100 c.c. under these conditions (Table IX). Again, owing to the small numbers of observations, I cannot give “normal” figures for the “urea concentration factor”; but so far, in 40 patients with a urea concentration (urine) over 2 per cent., the highest has been 72 and the lowest 18 (Table IX). These results seem to support the hypothesis that urea is a “no-threshold” substance.

As has been emphasised elsewhere (Harrison, 1921), in estimating renal efficiency it is necessary to weigh up all the evidence, both clinical and laboratory. After studying U.C.F.’s it is not surprising that it has been found clinically that the urea results are not absolute. For example, W. S— died from uræmia within 48 hours of cystoscopy; his urea concentration (urine) was 1·29 per cent. (urine 120 c.c. second and third hours combined) three days before the examination. On the other hand, R— is still alive several months after decapsulation of both kidneys, with a urea concentration shortly before the second operation of 1·18 per cent. (urine 70 c.c.). Again it is probably unsound to state that “. . . In no case should an operation necessitating a general anæsthetic be undertaken on any patient whose blood contains 100 mgrm. or more of urea” (MacLean, 1921). In some cases the conditions under which the estimation is made may profoundly affect the result; and the other findings (clinical and laboratory) may not contra-indicate operation in a few instances, in spite of high blood-urea. Thus McK— had a blood-urea of 100 mgrm. (low protein diet, no urea by mouth), and yet suprapubic cystotomy for malignant prostatic disease (under a general anæsthetic) was quite successful, the patient being discharged from hospital with a permanent drain, whereas another patient, L—, with a blood urea of 37 mgrm. (full hospital diet), died from uræmia three days after prostatectomy. “Rules,” such as the one quoted above, may be fully justified in the majority of cases, but a small minority of patients may suffer as a result of their too rigid application,

TABLE X.—*Examples of Nephritis with Edema Treated with Urea.*

No.	Urea retention.	Urea per cent. and c.c. of urine at end of—		Blood urea (mgrm. per 100 c.c.) (No urea by mouth.)	Edema before treatment.	Dose of urea. (Grammes.)	Duration of urea treatment.	Results and remarks.
268	Not tested	...	3.16; 90	...	+++ Could scarcely open eyes +++	16 daily	2 weeks	Edema almost completely disappeared in forty-eight hours.
133	...	2.13; 105	2.39; 60	...	+++	15 b.d.	4 "	Edema disappeared in three weeks.
166	Present.	1.41; 120	1.51; 90	59	+++	15 "	2 "	Same case as 133 with return of edema after two months at home. Edema disap- peared in one week.
138	Slight	0.73; 60	1.02; 110	45	+++	15 "	8 "	Edema much reduced by urea, and completely dis- appeared after a further two months on full protein diet.
221	Considerable.	1.49; 60	1.52; 60	88	++++	15 "	3 "	Urea no effect. Perhaps dose not large enough. Edema slightly increased in spite of the urea.



and occasionally it may be forgotten that the converse does not necessarily hold.

*Value of urea tests in prognosis.*—Though the tests are not “absolute,” repeated estimations at intervals, under standard conditions, on any given patient may be most helpful in following the progress of the disease (Table VII), although it is fair to question whether small variations have any clinical meaning. Obviously urea tests are but one link in the chain of evidence upon which a prognosis should be based.

*Value of urea tests in treatment.*—Whenever urea is used in the treatment of oedema in nephritis (MacLean and Russell, 1920; MacLean and de Wesselow, 1919) it would seem useful to perform urea tests beforehand. When chloride retention is unaccompanied by urea retention the treatment may be expected to work well. In one case (Table X, No. 268) urea worked like a charm, the oedema almost completely disappearing in 48 hours. But usually, as pointed out by the same authors, it is necessary to continue the urea (15 grm. twice daily) over a period of weeks or months. Frequently, in my experience, chloride retention is accompanied by urea retention. In such cases it would be expected either that urea would be of no avail, or that very large doses would be necessary to reduce oedema. In two instances when there was urea retention, oedema completely disappeared after a few weeks of urea together with the usual treatment for nephritis. In one patient the oedema slightly but steadily increased in spite of 30 grm. of urea daily for three weeks (Table X).

Occasionally patients complain that the repeated doses of urea cause nausea and headache, and rarely, vomiting follows. I find that autoclaving the mixture of urea 15 grm., tincture of orange 1 c.c. and water to 100 c.c. makes the taste slightly less unpleasant, and also stops the growth of a fungus which otherwise may occur if the solution is kept for a few days.

#### SUMMARY.

When applying the provocative urea concentration test:

- (a) It is advisable to withhold fluids for at least six hours before the test.
- (b) It is safest to measure the volume of urine passed each hour, even though the urea be not estimated in all samples. A volume greater than 150 c.c. (first hour), or 100 c.c. (second or subsequent hours), generally signifies excessive diuresis when a result below 2 per cent. does not necessarily imply an inefficient kidney. The test should be repeated and controlled by estimating the blood urea.
- (c) Occasionally it may be impossible to bring diuresis within these limits. Other tests are then essential. In one case of diabetes insipidus diuresis was controlled by 1 c.c. of pituitrin subcutaneously.
- (d) 15 grm. may not always be sufficient to provoke a concentration greater than 2 per cent., although the kidneys be efficient.
- (e) Proteinuria exceeding 0.4 per cent. (Aufrecht) may introduce a large error.

Blood-urea estimations may be misleading (*e.g.* before operation) if the nature of the diet is not considered.

By simply combining the urea-concentration test (15 grm. by mouth) and

the blood-urea estimation, the former is checked and the latter is standardised. The "urea concentration factor" (U.C.F.) so obtained is defined thus:

mgram. urea per 100 c.c. urine passed during third hour

mgram. urea per 100 c.c. blood obtained in middle of third hour.

Determinations of U.C.F. indicate that sometimes a "normal," or almost "normal" urea concentration (urine) may be due to a high blood urea, and illustrate the importance of estimating simultaneously the urea concentration in blood and urine.

The "normal" values for the urea concentration test (urine), the blood urea and the urea concentration factor are discussed.

The use of the urea tests in the prognosis of renal disease and in the treatment of œdema in nephritis is briefly mentioned.

I wish to thank the members of the staff at King's College Hospital for the opportunities of performing the above tests on their patients.

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## EXPERIENCES WITH THE SCHICK TEST AND ACTIVE IMMUNISATION AGAINST DIPHTHERIA.

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AFTER a preliminary investigation into the value of the Schick test and active immunisation against diphtheria (Copeman, 1921), the Ministry of Health instituted a more extensive test at the Mitcham Poor-Law Schools with the consent of the Guardians. In the present paper the immunological results are described.

The Mitcham schools contain a resident population of rather over 300 children between the ages of three and sixteen years. All, until quite recently, not only lived, but received their education, in one or other of the three adjoining institutions. The weekly rate of admission and discharge is small, and the population is thus a very stable one. Prior to November, 1920, these schools had been practically free from infectious disease, but since then they have been affected with scarlet fever and diphtheria almost continuously. No case of diphtheria has, however, been notified since August 4th, 1921.

The general scheme of work included a routine swabbing of all children and testing the bacilli isolated for virulence. The Schick test was applied in every case and the positives immunised with toxin-antitoxin mixtures, the effect of this being judged by re-testing by Schick's method at a later date.

### BACTERIOLOGICAL EXAMINATION.\*

All the children in the school and annexed infirmary, numbering 329, were swabbed on two separate occasions. The first set of swabs, hereinafter referred to as Series I, was taken eight weeks before the second set, called Series II. Children found on either occasion to be harbouring bacilli morphologically resembling *B. diphtheria* were re-swabbed until three consecutive negatives were obtained.

\* The whole of the technical work was planned and carried out by the staff of the Wellcome Physiological Research Laboratories, the bacteriology being done by one of us (A. J. E.), Dr. Okell and Miss Baxter, the examination of the blood samples for antitoxin and the control and testing of the Schick toxin and toxin-antitoxin mixtures by A. T. G. and Miss Allen, the Schick testing and the inoculations with toxin-antitoxin mixtures by R. A. O'B.

In all some 1400 swabs were examined by cultural methods. The following results were obtained :

*No. of Cases Diagnosed as "M.D." in Series I.*

"M.D." = bacilli morphologically resembling *B. diphtheriæ* in smear from "Loeffler" overnight culture.

"M.D." throat only . . . . .	1
"M.D." nose only . . . . .	8
"M.D." nose and throat . . . . .	9
Total positive . . . . .	18
Total swabbed . . . . .	329
Percentage positive . . . . .	5.47

*Cultures of B. diphtheriæ Isolated in Series I.*

	"M.D." diagnosis.	Cultures isolated.	Sugar reactions.		Virulent.	Avirulent.
			Correct.	Not done.		
Throat . . . . .	1	1	1	0	1	0
Nose . . . . .	8	3	1	2	2	1
Throat and nose . . . . .	9	4*	3	1	2	2

"Correct" sugar reactions were acid production in glucose, but not in saccharose. Virulence was tested by the intracutaneous method checked by the subcutaneous (Eagleton and Baxter, 1921).

\* Four cultures from two patients who both harboured virulent and avirulent strains, but at different times.

*No. of Cases Diagnosed as "M.D." in Series II.*

"M.D." throat only . . . . .	1
"M.D." nose only . . . . .	3
"M.D." nose and throat . . . . .	2
Total positive . . . . .	6
Total swabbed . . . . .	327
Percentage positive . . . . .	1.8

*Cultures of B. diphtheriæ Isolated in Series II.*

	"M.D." diagnosis.	Cultures isolated.	Sugar reactions.		Virulent.	Avirulent.
			Correct.	Not done.		
Throat . . . . .	1	0	0	0	0	0
Nose . . . . .	3	2	2	0	0	2
Nose and throat . . . . .	2	2	2	0	0	2

*Results of Series I and II Compared.*

	"M.D." diagnosis.	Cultures isolated.	Sugar reactions.		Virulent.	Avirulent.
			Correct.	Not done.		
Series I . . . . .	18	8	5	3	5	3
Series II . . . . .	6	4	4	0	0	4
Common to both . . . . .	3	2	2	0	0	2
Total . . . . .	21	12	9	3	5	7



The swabs in Series I were taken at the end of a series of cases of clinical diphtheria, when opportunities for infection of throats were fairly plentiful; one would naturally expect that the later series, taken at a date two months further away from the existence of manifold opportunities for infection, would show a lower reading of infection, and this is what we found.

With regard to the difficulties of isolation, one or two short notes may be made. The digestion of Loeffler's medium by organisms occurring in the throat and nose, but especially the latter, is one fruitful source of trouble. Another great difficulty consists in the fact that "M.D." may be present in very small numbers indeed. We do not think it is going too far to say that the ease with which *B. diphtheriae* can be isolated from the nose or throat is a measure of the abundance of this organism in the nose or throat, and therefore, most probably, of the danger to the community arising from the patient under examination.

In our opinion it follows that the percentage of cases from which virulent *B. diphtheriae* can be isolated is more important than a carrier rate based on morphological criteria, which disregard the pathogenic power of the organisms found and the number present.

In support of this view, and as a contrast to the results in these two series, the following brief account of some work that has been largely contemporaneous, and so has acted as a control, will be of interest. One hundred and fifty convalescents from clinical diphtheria were swabbed at different intervals after the disease. In 14 "M.D." was diagnosed, and although one swab only from each case was submitted to us, from 13 out of those 14 virulent *B. diphtheriae* were isolated.

In Series I five virulent carriers were found, one of them being convalescent from clinical diphtheria at the time of swabbing. The most persistent gave an initial "virulent"; subsequent cultures were "avirulent." In Series II no "virulent" carriers were found.

There had been a series of cases of clinical diphtheria in this institution, and it was to be expected that one or more carriers of virulent bacilli, easily isolated and continuously excreted, would be found. No such carrier was, however, discovered. But our examination was not made at the height of the epidemic, and we know from general experience (*cf.* also Hartley and Martin, 1920) that most convalescents rapidly become clear of bacilli. It may be that one of the five "virulent" carriers (or, less probably, one of the seven "avirulent" carriers) was the cause of the epidemic, no evidence having been obtained that the infection was derived from a source outside the institution.

#### SCHICK TESTING.

*Toxin.*—The same toxin was used throughout. Fresh dilutions were made for each day's work, and the potency of the diluted toxin remaining over was tested on guinea-pigs (Glenny, Allen and O'Brien, 1921). We adopted the original Schick formula as used by Park and Zingher, 0.2 c.c. containing  $\frac{1}{10}$  guinea-pig M.L.D. being injected. In one group of about thirty children in which the first Schick test had given rise to some slight uncertainty, it was repeated, the original Park formula being used simultaneously with Zingher's later modification, in which 25 per cent. more toxin is used on the left arm

and 50 per cent. extra toxin in the heated control on the right arm. In this small group of cases we could not find that the Zingher modification gave any clearer readings than the original formula.

*Readings.*—In a discussion of the reaction it is necessary to have concise descriptive signs or terms which should convey as much information as possible. We think this question is so important that we append a short table of explanation of the conventions that we would suggest.

*Schick Test Nomenclature.*

Type of reaction.	Written description of reaction.	Verbal description of reaction.	Description of patient.	Description previously in use.
1 .	—	Negative	Immune = I.M.	Negative.
2 .	— (ψ)	Negative and pseudo	Immune (pseudo reactor) = I.M.P.	Pseudo.
3 .	+	Positive	Non-immune = N.I.M.	Positive.
4 .	+(ψ)	Positive and pseudo	Non-immune (pseudo reactor) = N.I.M.P.	Combined.

The readings were made daily up to five or seven and occasionally thirteen days. If one decides to make one reading only, the most satisfactory is that made from the fourth to the seventh day after the injection.

The results of our tests are set out in Table I. The percentage of positive reactions obtained for this group of children, between the ages of three and sixteen, correspond closely with that published by Park.

TABLE I.—*Schick Test Results at Various Ages.*

Age in years.	Per cent.				
	Immune and immune (pseudo reactor).	Non-immune.	Total.	Immune and immune (pseudo reactor).	Non-immune.
3-4 .	9 .	3 .	12 .	75·0 .	25·0
5-6 .	20 .	8 .	28 .	71·4 .	28·6
7-8 .	30 .	12 .	42 .	71·4 .	28·6
9-10 .	39 .	22 .	61 .	63·9 .	36·1
11-12 .	55 .	21 .	76 .	72·4 .	27·6
13-14 .	51 .	23 .	74 .	68·9 .	31·1
15-16 .	23 .	13 .	36 .	63·9 .	36·1
Total .	227	102	329	69·0	31·0

*Technique and reading of results.*—So far as the technique is concerned, we have but little to add to the excellent description in the publications of Park and Zingher. The needle and syringe must work without any defect; the slightest bluntness of the needle or leak of the plunger at the junction of the needle results in unsatisfactory work. We used a Burroughs Wellcome No. 1 dental needle with a 1 c.c. all-glass syringe, and a 1 c.c. long



tuberculin syringe; in one series a "Record" 1 c.c. syringe was used. It is most convenient to use two syringes, one for the toxin, the other for the control; these should be of the same make, with the plunger working equally well, and the needles should be equally sharp, otherwise a slight difference in the depth at which the intradermic injection is made may occur, with resultant blurring of the readings, particularly those of the first day. The control syringe may be identified by a rubber band fixed around the barrel.

Readings were made daily in most of the cases up to five or seven days, each day's reading being made without reference to the previous readings. Wherever there was any discrepancy the Schick test was repeated. Fifty-five of the children were thus re-tested because some slight doubt or discrepancy had occurred in the course of the first test (Table II).

TABLE II.—"Doubtful" Schick Results among 329 Children Tested.

First Schick test.		Second Schick test.	
Readings regarded as probably:			
— & — (ψ)	+ & + (ψ)	— & — (ψ)	+ & + (ψ)
44		44	
	11	4	7
Total 44	11 = 55	48	7 = 55

Consideration of these cases shows that when a reading is doubtful, it proves on further investigation in the great majority of cases to be negative or "negative (and pseudo)." When deciding that a given reaction is "negative (and pseudo)," one has always present in one's mind a slight fear that the reaction may be "positive (and pseudo)." Fortunately these latter reactions are rare. Of the 329 children only two showed a "positive (and pseudo)" reaction, *i. e.* on the right arm a reaction which, although smaller, resembled the left in depth of colour and degree of desquamation.

Table III gives details of the four cases in Table II in which differences were obtained on re-testing.

TABLE III.—*Apparent Discrepancies.*

First test.	Second test.	Blood sample.
1. Positive (and pseudo)	Negative (and pseudo)	More than $\frac{1}{30}$ unit of
2. Positive	Negative (and pseudo)	antitoxin per c.c. of
3. Positive	Negative (and pseudo)	blood found in every
4. Positive (and pseudo)	Negative (and pseudo)	case.

On referring to the readings we had recorded in the first test, we found in every case that the final entry, on which the child was classed, recorded a "very faint" or "very, very faint" stain on the left arm, greater in size than on the right, and that some of the readings had suggested a "negative (and pseudo)" reading. With the experience gained in these cases and the help supplied towards the interpretation of doubtful readings by the determination of antitoxin in the patient's blood, we should, with similar readings now, return the case as "immune (pseudo reactor)."

*Blood samples.*—Our confidence in the ultimate accuracy of our classification of the children as immune or non-immune is founded partly on the results of the repeated Schick tests in "doubtful" or difficult cases, but to a greater extent on the interpretation of the titration of antitoxin in the blood of individual children by the Römer method (Glenny and Allen, 1921). This we regard as a very important part of our investigation.

Many observers state that an antitoxin content per c.c. of blood of less than  $\frac{1}{30}$  unit of antitoxin will fail to neutralise the toxin injected in the Schick test and so make the readings positive, while, with a greater antitoxin content, a negative Schick reaction will result. Our own experience in several hundred tests has hitherto afforded no ground for disagreement with this statement.

Sixty-six blood samples were taken and the antitoxin titrated. The findings are shown in the table.

TABLE IV.

Antitoxin per c.c. of blood.	No. of cases.	
5-20 units . . . . .	7	} 8 of these children had had toxin- antitoxin mixtures injected.
2-5 " . . . . .	8	
1-2 " . . . . .	15	
$\frac{1}{2}$ -1 unit . . . . .	9	
$\frac{1}{5}$ - $\frac{1}{2}$ " . . . . .	9	
$\frac{1}{100}$ - $\frac{1}{10}$ " . . . . .	5	
? $\frac{1}{1000}$ " . . . . .	5*	
Less than $\frac{1}{2000}$ unit . . . . .	8	

*Note.*—In many instances the sample of blood for titration of antitoxin was not taken until approximately a week after the Schick test was done.

Recent experiments on animals (yet to be published) show that the minute amount of toxin used in the Schick test is sufficient, under certain circumstances, to cause the development of such a degree of immunity that an animal which has just given a non-immune Schick positive result, will, a week or two later, when the test is repeated, give an immune negative response. This may possibly be the explanation of some of the discrepancies recorded.

It is probable that these results will be confirmed on the human subject, but the point will be dealt with in a subsequent paper.

*Error on first day's reading.*—It is obviously of great importance in the presence of an epidemic of diphtheria to be able, if possible, to make a decision that the given patient is immune or not within the first twenty-four hours of the Schick test. We did in all approximately 400 Schick tests. In four instances the first day's reading was entered as "negative," whereas later readings showed that the reaction was "positive." First day's readings of the reaction in eight children were "positive," but the later readings showed clearly that the reaction was "negative" and the children therefore immune. In nine instances the first day's reading entered as "doubtfully" positive or negative differed from the final reading. Thus an actual error was made in

\* All of these five sera, when injected intradermically without toxin into guinea-pigs, gave some reaction; it was, therefore, difficult to estimate the antitoxin content with the small amounts of serum available.



twelve cases and a dubious but erroneous reading in nine, *i. e.* 21 in all. In 400 tests, therefore, a decision based on the first day's reading proved erroneous in 5 per cent. of the cases.

"*Carriers.*"—The following table gives the results of the Schick test and examinations of the blood of children who harboured "morphological diphtheria bacilli." It is to be noted that the carriers of virulent bacilli possessed a fairly high degree of immunity.

TABLE V.

	Virulent cultures isolated.	Avirulent cultures isolated.	"Hofmann" isolated.	"M.D." not isolated.
	5	7	4	6
Schick test positive	0	1	2	1
Schick test negative	5	6	2	5
Blood samples examined.	5	1	—	3
Antitoxin content per c.c.	4, 1 unit	< $\frac{1}{2000}$		1. < $\frac{1}{2000}$
	1, $\frac{1}{50}$ unit*			2. $\frac{1}{2}$ —1
				3. 2—5

*Note.*—In some of these instances the sample of blood for titration of antitoxin was not taken until approximately a week after the Schick test was done.

#### ACTIVE IMMUNISATION.

All children giving a positive Schick reaction, 102 in all, were given three weekly doses of 1 c.c. of toxin-antitoxin mixture (Park-Zingher formula and American official standard). As a precautionary measure, in most cases a preliminary injection of 0.05 c.c. had been given.

*Reactions.*—The results were reassuring. Of the 102 children 17 were reported to the nurse (one child twice). Eight had a temperature of 102°, four of 101°, and two of 99°. Though this number (17) seems large, the reaction was so slight in all but three of the children that they did not wish to stay in bed for a whole day. One of the remaining three wished to stop in bed for two days.

W. G—, one of two that were ill, had a temperature of 102°, and was distinctly ill for three days after her first dose of 0.05 c.c. A week later she received 0.01 c.c., and at intervals of five days 0.05 c.c., 1 c.c., 1 c.c., 1 c.c. She remained quite well throughout.

It is therefore probable that the initial illness was due to some other cause than the injection of the mixture.

The other child, A. B—, vomited immediately after the first injection, and was ill for two days. Inquiry showed that she had had a pork dinner a few hours before the injection, and had suffered from abdominal pain before the injection. Unfortunately this was not known to us.

The constitutional reactions were slight. Local reactions, on the other hand, were at first rather alarming. In approximately half of the cases

\* Patient G. M— referred to under "Results of Immunisation" (p. 49).

a large flushed area varying from 2 in. by 1 in. to even 6 in. by 3 in. appeared, but rapidly cleared up after the first twenty-four to forty-eight hours. We were very disturbed until we learned by experience that a large, angry-looking swelling would not prevent a boy from playing football two days later or a girl from skipping on the first day after the injection, and that it did not interfere to any great extent with sleep.

*Results of Immunisation.*—Approximately eleven weeks after the conclusion of the course of immunisation the whole of the children in the school were again subjected to the Schick test.

Of the 227 children who had been classified by the first Schick test as immune, 203 remained in the school; of these 201 again gave negative or negative (and pseudo) reactions, while two children showed positive reactions. Samples of blood were obtained from these two, and neither contained any antitoxin. Both children had had diphtheria, and had been removed to hospital, where they were given antitoxin shortly before the first Schick test was done. It appears probable that the first Schick test, which in one child was undoubtedly negative and in the other (G. M—) had been read as "pseudo (with a faint possibility of positive)," had been influenced by some remnants of the antitoxic serum which had been injected in hospital. Before the second Schick test was performed, some three months later, the last remnants of the horse antitoxic serum had been excreted, and the Schick results were therefore positive.

Of the 102 children who had given a positive reaction and had been inoculated with a toxin-antitoxin mixture, 99 remained and were re-tested. Two still gave a clear positive reaction; samples of their blood were tested; no antitoxin was present in either. These children will be re-inoculated.

#### CONCLUSIONS.

(1) In a residential school of 329 children, amongst whom a recent epidemic of diphtheria had occurred, eighteen (6 per cent.) carried morphological *B. diphtheriae* in throat or nose. At the first swabbing, at the end of the epidemic, 5 per cent. of carriers were found; at the second, two months later, 2 per cent. From these eighteen children twelve cultures were isolated, of which five were virulent.

(2) The later swabbings from these five carriers showed either avirulent organisms or no "morphological diphtheria" bacilli.

(3) On the grounds of a bacteriological examination of a large number of cases of diphtheria, carriers and convalescents, the following suggestions are tentatively put forward:

(i) That the "avirulent carrier" is of no importance epidemiologically.

(ii) That the danger of a carrier of virulent bacilli is, at the time of examination, proportionate to the number of virulent bacilli present in throat and nose, and, therefore, to the ease with which the virulent bacilli can be isolated.

(4) Of 329 children, aged from three to sixteen, 102, *i. e.* 31 per cent., gave a positive Schick reaction; 95 per cent. of the readings made on the first day proved to be accurate.

Of the 227 children who had given a negative response when first



Schick tested, 203 remained in the school eleven weeks later. Of these, 201 on being re-tested again gave a negative or a negative (and pseudo) response, thus confirming the decision made two months previously. Two showed a positive response at the second test; these two children had had antitoxin injected shortly before the first test was made.

(5) These 102 children were inoculated with toxin-antitoxin mixtures (Park and Zingher formula). Local reactions occurred in about one-third of the children; though in some of these the area of inflammation was large, the activities of the children were but little interfered with. Constitutional reactions were slight in all but two of the children.

(6) Of the 102 children, 99 were still present in the school eleven weeks later when the Schick test was repeated. Two gave an undoubtedly positive response, the remainder a negative or negative (and pseudo) reaction; 98 per cent. were therefore immune.

We have much pleasure in thanking Dr. Manby for interesting the Guardians in the test, and Dr. Morrish, the Medical Officer; Mr. Drury, the Superintendent of the Schools; Mrs. Drury, the Matron, and the Infirmary Sister, for the valuable assistance afforded by them, especially in arranging practical details in connection with the work.

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## THE CARBOHYDRATE METABOLISM OF SURVIVING MOUSE TISSUES AND TUMOURS.

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*From the Laboratories of the Imperial Cancer Research Fund.*

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THE present communication may be regarded as a continuation of a paper by the author and W. H. Woglom (1920), published in the first volume of this journal. On that occasion a method was described of ascertaining the gaseous exchange of surviving tissues of the mouse, and especially of arriving at the ratio of the carbonic acid output to the oxygen intake—that is, the respiratory quotient. The reader is referred to that paper for the details of the procedure adopted, which may here be briefly described as consisting in the measurement of the changes of volume observed in an air space containing a known amount of finely-divided tissue or tumour suitably disposed to facilitate the respiration of the cells. The apparatus employed was a modification of the well-known Barcroft blood-gas analyser, and the same instruments, three in number, have been used for the experiments about to be recorded.

A series of observations done in the above manner gave differences, which we regarded as significant, in the case of the several normal tissues examined, namely kidney, liver, mamma, submaxillary gland and embryo. Six strains of transplanted tumours gave still greater differences in their average respiratory quotient, and the quotient of the more rapidly growing tumours was found to be higher than that of the more slowly growing tumours. Interpretation of these results in terms of the food-stuffs being burnt led to the general conclusion that the rapidly growing neoplasms katabolised more carbohydrate, whilst those of slower growth burnt mainly fat. One notable exception to this generalisation was found with a tumour of medium rate of growth, which, nevertheless, gave the highest quotient found. This particular strain was distinguished in another respect from its fellows, as its cells always contained a considerable amount of glycogen. Consideration of these facts at once raised the further question: Does a low respiratory quotient mean that a tumour strain can only consume carbohydrates to a limited extent, or does it only mean that in the parenchyma there is no readily available supply? A comparison of the results obtained with normal kidney and liver showed that with an abundant supply of glycogen in the case of liver, the quotient found was appreciably lower than that of kidney which was free, at least histologically, from glycogen. Liver, also, did not react in the same way to its carbohydrate content as the glycogen-bearing tumour mentioned above. Further experiments were necessary to test the question, and these took the



line of adding small amounts of different sugars to the tissue emulsion before placing in the respirometer bulb.

The sugars tested were in all cases made up into a solution containing two parts pro mille. As diluent, Ringer solution could not be used because of the bicarbonate present, and a 0·85 per cent. saline solution made with tap water was adopted. The tissue emulsion was spread in a thin uniform layer on one side of a rectangular slip of tissue paper which was then floated on a small pool of sugar solution. The emulsion adheres to the filter-paper, and allows the excess of solution to drain off. It was next placed on a slip of glass, and introduced into the respirometer bulb, where the further treatment was exactly similar to that already described in the previous communication.

The taking up of sugars by surviving tissues such as the mammalian heart has been the subject of investigation by a number of experimenters, and the points of more immediate interest will be found in the papers by Locke and Rosenheim (1904), Rohde (1910), Neukirch and Rona (1912), Maclean and Smedley (1913), Evans (1914), and Starling and Evans (1914). Usually the consumption of glucose has been estimated from analysis at intervals of the perfusing fluid. A useful summary of this work is given by Starling and Evans, who have further shown that the results obtained by this method agree with the calculation made on the results of gaseous metabolism experiments on the heart-lung preparation of the dog. Evans found that the addition of glucose to the circulating fluid raised the respiratory quotient by increasing the  $\text{CO}_2$  output, but a greater increase could be obtained by previous carbohydrate feeding of the living animal. It was especially the work of these two authors which encouraged the writer to persevere in the attempt to analyse the carbohydrate metabolism of transplanted tumours on the same general principle.

A number of preliminary observations were made with mouse kidney emulsion with the especial object of ascertaining the effect of the saline solution, and further of finding a suitable strength of sugar. A series of six

TABLE I.—*Mouse Kidney. The upper figures in each observation are the respiratory quotients; the lower figures (in brackets) give the oxygen consumption in c.c. per kilogramme-minute.*

							Average.
Glucose . . .	0·966	0·943	0·941	0·952	0·932	0·940	0·946 $\pm$ 0·005
	—	—	(20·9)	(20·7)	(20·8)	(18·0)	
Lævulose . . .	0·928	0·920	0·914	0·918	0·957	0·951	0·931 $\pm$ 0·007
	(22·0)	(21·8)	(25·2)	(24·0)	(25·5)	(20·9)	
Galactose . . .	0·888	0·886	·844	—	—	—	0·873
	(21·9)	(20·7)	(17·7)				
Maltose . . .	0·919	0·939	0·915	0·949	—	—	0·930
	(19·4)	(27·1)	(25·7)	(25·7)			
Saccharose . . .	0·895	0·898	0·891	0·872	—	—	0·889
	(14·7)	(22·1)	(22·4)	(22·8)			
Lactose . . .	0·933	0·896	0·877	0·886	—	—	0·898
	(22·4)	(24·6)	(24·5)	(19·7)			
Saline . . .	0·871	0·863	0·883	0·868	0·880	0·868	0·877 $\pm$ 0·006
	(21·2)	(18·4)	(16·5)	(21·1)	(17·7)	(17·1)	

experiments was carried out with kidney emulsion in saline, and the respiratory quotients determined will be found in the bottom row of Table I. The mean quotient is  $0.877 \pm 0.006$ , and approximates very closely to the figure previously found for untreated kidney emulsion, namely  $0.883 \pm 0.002$ . The rate of absorption of oxygen varies fairly widely, but does not betray any deleterious action by the saline solution, which can be regarded as suitable for the purpose in every way. Glucose was next added to the saline in increasing amounts, and a strength of two parts pro mille was found to give a distinct rise in the respiratory quotient. This strength was adhered to throughout the series of experiments, and was also adopted for the other five sugars tested. Gravimetric determination of the amount of sugar solution imbibed by the filter-paper and tissue emulsion showed that enough sugar was taken up to cover the needs of the cells over the duration of the experiment. The sugars tested were only those of physiological importance.

On Table I the respiratory quotients and the oxygen consumptions are given for kidney emulsion. For lucidity the average respiratory quotients are given in Fig. 1 as abbreviated columns. From this it will be seen that the addition of glucose, levulose or maltose has raised the respiratory quotient to an appreciable extent. The deviation observed with galactose, saccharose and lactose is too small to be regarded as significant. It is a difficult matter to decide the figure to be exceeded before the result can be looked upon as a definite answer to the question as to whether the sugar is being consumed or neglected. In arriving at a conclusion such different factors as the consistent

results given by any tissue and the margin for experimental error in the method used have an important bearing. Starling and Evans (1914) concluded that only a rise of more than .05 in the mean quotient could be regarded as indicating an increased consumption of sugar by a diabetic heart to which pancreas extract had been administered. The addition of glucose, levulose and maltose to mouse kidney has increased the mean quotient by a trifle more than .05, and the evidence in favour of their consumption is quite conclusive. Since the respiratory quotients found by the method are remarkably uniform, one would be justified in regarding a much smaller variation as of importance.

The rate of oxygen consumption is also given in Table I, and it will be seen that this shows the usual wide variations found by this method, which are attributable to different degrees of fineness of the kidney emulsion, and varying degrees of thinness of the layer spread on the piece of filter-paper. In general there is no distinct alteration of the rate of oxygen consumption,

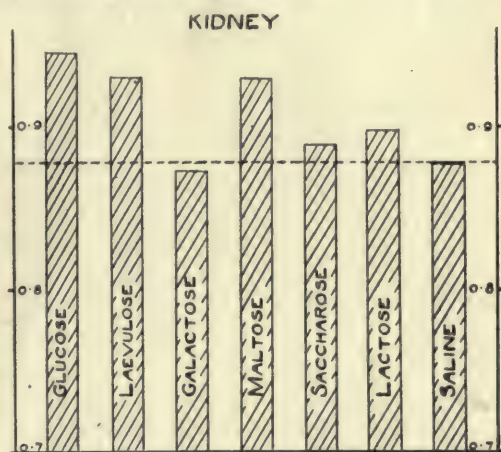


FIG. 1.—Diagram to show the effect of sugars on the respiratory quotient of kidney emulsion.



and the increased quotient obtained by using glucose, lævulose and maltose must be ascribed to an actual increase of the  $\text{CO}_2$  given off. Neukirch and Rona (1912) found that the perfused rabbit heart used glucose and galactose, but not lævulose, saccharose, lactose or maltose. Similar results were got by Maclean and Smedley (1913), excepting only that in the case of the dog lævulose was retained in varying quantities. It would appear, therefore, that minced mouse kidney behaves differently towards maltose and lævulose. Lævulose prepared from inulin gives the same rise in quotient with mouse kidney, so that its consumption must be regarded as real and not attributable to the presence of glucose as an impurity.

A number of experiments were next done with minced liver, and the results are given in Table II. The respiratory quotient for this tissue varies

TABLE II.—*Mouse Liver. The upper figures are respiratory quotients; the lower (in brackets) give oxygen consumption in c.c. per kilogramme-minute.*

Glucose	0.952 (9.4)	0.769 (7.0)	0.893 (8.2)	—	—	—	—	—	—
Lævulose	0.830 (8.9)	0.850 (7.3)	0.889 (7.8)	—	—	—	—	—	—
Galactose	—	—	—	—	—	—	—	0.778 (8.4)	0.722 (8.7)
Maltose	—	—	—	0.855 (8.2)	0.866 (7.4)	0.790 (7.8)	0.862 (10.8)	—	—
Saccharose	—	—	—	—	—	—	—	0.813 (7.9)	0.772 (10.0)
Lactose	—	—	—	0.856 (8.8)	0.841 (8.4)	—	0.847 (9.2)	—	—
Saline	0.801 (8.0)	0.829 (7.0)	0.850 (7.7)	0.794 (7.8)	0.861 (8.0)	0.734 (8.1)	0.818 (9.0)	0.817 (8.4)	0.765 (10.0)

rather widely, and it was necessary to vary the procedure slightly to ensure adequate controls. The emulsion was divided into three portions, one of which was soaked in the saline solution serving as control medium, whilst the other two portions were treated with different sugars. In Table II the figures are to be read vertically, those at the bottom being the saline control to the sugars above. In the subsequent tables for the four tumours examined the same arrangement has been adopted. The number of experiments with liver tissue is fewer, and the results are less clear than with kidney, but in general they bear a similar aspect. Glucose, lævulose and maltose appear to be burnt, whilst galactose and saccharose are untouched. The slight indication given by kidney of the consumption of lactose appears again in two out of three observations with liver. The raising of the quotient by glucose, lævulose and maltose is not so extensive or consistent as with kidney emulsion. The peculiar rôle played by the liver in normal metabolism is sufficient to explain its comparative neglect of the various sugars added in these experiments.

Four different tumour strains have been examined, three carcinomata and one sarcoma. Two of the carcinoma strains, 72 and 155, are of slow growth, whilst the third, strain 63, grows rapidly, and the sarcoma, strain 37, is the

most rapidly growing of all. Table III contains the data found with this sarcoma. The quotients are, in all cases, comparatively high, but the addition of glucose, lævulose or maltose results in a distinct raising. In two instances quotients slightly exceeding unity have been obtained. Galactose, saccharose and lactose have not affected the quotient.

With carcinoma 63 (Table IV) glucose and maltose give a distinct rise in the quotient, but in the two experiments with lævulose a rise in one is neutralised by a fall in the other. The same was found on adding galactose. A series of six experiments were done with lactose to settle, if possible, the significance to be attached to the small rise in the quotient observable on addition of this sugar. Five of the six showed varying rises, and one a slight fall. The average quotient for the lactose-treated emulsions was  $0.923 \pm 0.014$ , whilst the controls gave  $0.879 \pm 0.012$ . These averages speak in favour of a slight consumption of the lactose added, the increase of the quotient being rather more than can be fairly ascribed to experimental error. The mean rise of the quotient after adding lactose is 5.2 per cent., with a mean error of 2.1.

A more slowly growing adeno-carcinoma, strain 72, gave in the control experiments a quotient of  $0.769 \pm 0.027$ , but the addition of glucose or maltose raised the quotient to about or even over unity (Table V). The limited number of observations do not allow any definite conclusion to be drawn with regard to the other four sugars tested, but in any case one misses the marked rise noted with glucose and maltose. Another tumour strain, adeno-carcinoma 155, very similar to the preceding, but growing even more slowly, reacted to the addition of carbohydrates in almost the same way (Table VI). The control experiments gave a mean quotient of  $0.786 \pm 0.008$ , which is higher than the quotient found previously for this strain. A temporary increase in the rate of proliferation of the strain is the most probable explanation of this rise in the quotient, for comparison of the rate of growth of any tumour in a series with its respiratory quotient reveals this association. It will be seen from Table VI that glucose and maltose are attacked, whilst lævulose and lactose show only a small rise in the quotient, and the results from galactose and saccharose are negative.

TABLE III.—37 Sarcoma. Figures arranged as in Table II.

Glucose	—	—	—	0.917 (4.3)	1.072 (6.2)	—	—	—
Lævulose	0.944 (9.8)	0.985 (6.3)	0.972 (4.9)	—	—	—	—	—
Galactose	0.836 (9.8)	0.784 (3.5)	0.954 (4.6)	—	—	—	—	—
Maltose	—	—	—	—	—	1.011 (7.2)	0.939 (6.7)	—
Saccharose	—	—	—	—	0.878 (6.9)	0.881 (7.6)	—	—
Lactose	—	—	—	0.851 (4.6)	—	—	0.835 (6.8)	—
Saline	0.852 (7.4)	0.847 (5.9)	0.945 (4.6)	0.846 (4.1)	0.904 (6.5)	0.842 (7.3)	0.854 (6.0)	—





The figures given in the preceding six tables do not easily lend themselves to the formation of a general summary, but a more comprehensive survey will be found in Table VII. In this table a rather arbitrary assignation of marks has been given to the various tissues, an increasing number of plus marks indicating a greater rise in the respiratory quotient from the addition of the respective sugars. The values given are :

TABLE VII.

	Kidney.	Liver.	37	63	72	155
Glucose . .	++	+	++	+++	+++	+++
Lævulose . .	++	+	++	—	+	+
Galactose . .	—	—	—	—	—	—
Maltose . .	++	+	+++	+++	+++	++
Saccharose . .	—	—	—	—	—	—
Lactose . .	+?	—	—	+	—	+?

These are at best only crude approximations, but they bring out in a more visible form the parallel behaviour of the different tissues and tumours. Glucose and maltose seem to be attacked by all tissues with equal facility, whilst galactose and saccharose are neglected. The burning of lævulose by kidney and 37 sarcoma is missed in 63 carcinoma, and only faintly indicated by the two adeno-carcinomata 72 and 155. This default on the part of strain 63 is compensated on the other hand by its combustion of lactose—a power which is indicated only by kidney and 155.

The addition of glucose to emulsions of the four different tumour strains has raised the respiratory quotient to about unity in all cases. This rise is particularly striking in the cases of the slowly growing strains 72 and 155, strains which ordinarily have low quotients of from 0·7 to 0·8. As glucose is the sugar transported by the blood-stream, the explanation of the lowness of the respiratory quotient in the case of the more slowly growing tumours is not a simple one. The present series of experiments show that the cells of these tumours are quite capable of dealing with an adequate supply of glucose, and there is not an anatomical difference between their blood supply and that of the tumours of a higher proliferation rate, such as might affect their comparative states of nutrition. In almost all transplanted tumours the blood supply is notoriously defective, and leads to early and wide-spread necrosis. The facts observed would appear rather to be explicable along the lines of postulating differences in the amount of available carbohydrate in the parenchymata of the various tumours. The gravest objection to the estimation of metabolism of surviving tissues by the present method is of course the failure to renew the supply of food-stuffs. This is met to some extent by shortening the duration of the experiment to a period during which no alteration occurs in the rate of oxygen consumption, so that one has good reason for presuming that there has not been an exhaustion of the available food supply. In view of all the observed phenomena, it seems reasonable to suppose that the cells of slowly growing tumours have a lesser amount of carbohydrate present in a readily available form.



## SUMMARY.

The low respiratory quotient observed with slowly growing transplanted tumours is not attributable to their incapacity of burning glucose. Mouse tumours and tissues appear capable of dealing with the common sugars in a very similar manner, provided the sugars are supplied directly. Differences in the quantity of sugar within the cells is a probable explanation of the variations in the carbohydrate metabolism of tumours growing at different rates.

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## THE WASSERMANN REACTION IN RELAPSING FEVER.

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WHILE carrying out routine Wassermann reactions at the Citadel Military Hospital, Cairo, I thought it would be of interest to examine the blood of cases of relapsing fever by the same method.

In looking through the literature only two references to this subject were found.

Korshun and Leibfried (1909), in a series of 50 cases, using syphilitic antigen alone in 23 cases, and both syphilitic and spirochætal antigens in 27, found that 28 cases (56 per cent.) were positive to the syphilitic antigen and 26 cases (96 per cent.) to the spirochætal antigen. They state that the difference is merely one of degree, and that there is no influence due to the duration and period of the infection.

In a more recent paper Fairley and Sullivan report (1919) on 32 cases of relapsing fever, and they state that in 10 cases during the pyrexial period 1 was positive, and in 22 cases in the apyrexial period there were 3 positive. The results were the same with the usual and the "ice-box" methods.

The observations recorded in this paper differ from the above in that the same cases were examined at different periods in order to follow the variations in the reaction during the course of the infection, and to demonstrate more clearly that the reactions were not due to syphilis.

The methods that were used were Emery's and Fleming's modifications of the Wassermann reaction. The cases were followed to determine how long after the disease the positive reaction remained and the effect of treatment on the reaction.

Out of 18 cases 11 were positive at some stage of the disease, but 6 of the 7 negative cases were only tested once. Excluding 2 of the positive cases in which the test was not repeated, it is seen that 3 out of 9 persisted during the period of observation (17 to 21 days), while all the others had become negative 8 to 13 days after the onset of the fever. Two cases were negative on first examination, but were found to be positive 3 and 6 days after onset (Table I).

From this evidence it appears probable that a transient positive Wassermann reaction may be found to be a constant phenomenon during the acute stage of relapsing fever. The transient character distinguishes it from the reactions due to syphilis. If the positive result is observed on every occasion in a particular patient, it may be suspected that he has syphilis also.

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TABLE I.

Case.	Date.	Approximate number of days since last attack.	Probable number of attacks.	Salvarsan, 0.6 grm., given.	Result of Wassermann reaction.
1	27 : 1 : '17	4 days	2		+
	31 : 1 : '17	8 days		27 : 1 : '17	-
2	27 : 1 : '17	1 day	2		+
	31 : 1 : '17	5 days	2		+
	7 : 2 : '17	12 days		1 : 2 : '17	+
	14 : 2 : '17	19 days			+
3	31 : 1 : '17	1 day	2		+
	7 : 2 : '17	8 days		1 : 2 : '17	+
	14 : 2 : '17	15 days			-
4	31 : 1 : '17	7 days	2		-
5	31 : 1 : '17	28 days	1	3 : 1 : '17	-
6	31 : 1 : '17	23 days	1	6 : 1 : '17	-
7	7 : 2 : '17	10 days	2	29 : 1 : '17	+
	14 : 2 : '17	17 days			+
8	7 : 2 : '17	5 days	2		+
9	7 : 2 : '17	8 days	2	3 : 2 : '17	-
10	14 : 2 : '17	Temp. still up	2		-
	21 : 2 : '17	6 days		20 : 2 : '17	+
	28 : 2 : '17	13 days			-
	28 : 3 : '17	41 days			-
11	21 : 2 : '17	7 days	1		+
	28 : 2 : '17	14 days		23 : 2 : '17	+
	7 : 3 : '17	21 days			+
12	21 : 2 : '17	4 days	2		+
	28 : 2 : '17	11 days		23 : 2 : '17	-
13	28 : 2 : '17	12 days	1		-
		Temp. went up next day			
	7 : 2 : '17	3 days	2		+
	14 : 3 : '17	11 days		8 : 3 : '17	-
14	21 : 3 : '17	3 days	2		+
15	24 : 3 : '17	Nil	2	Given during fever	-
	28 : 3 : '17	4 days	2		-
16	3 : 4 : '17	2 days			+
	11 : 4 : '17	8 days			-
17	11 : 4 : '17	4 days		10 : 4 : '17	-
18	13 : 4 : '17	Nil			-
		Temp. still up			

# THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY

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## THE EFFECT ON ARTERIAL HYPERTENSION OF INCREASED FLUID INTAKE.

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It has long been supposed that contraction of the arterioles resulting in increased peripheral resistance is induced by certain decomposition products of protein, either produced in the tissues or absorbed from the intestine. In more recent years, experimental evidence has appeared in support of this supposition. In an investigation carried out here, on the influence on protein metabolism of a sudden increase in the amount of water passing through the system, results were obtained which suggested that the increased ingestion of water affects the metabolism of protein in such a way that the formation of pressor substances is reduced. The series of experiments of which some are recorded here were therefore undertaken to determine what influence increased water intake has upon the blood-pressure.

### EXPERIMENTAL METHODS.

In each experiment, a number of readings of the systolic and diastolic blood-pressure were taken in a preliminary control period of two or three days. During this period, the subject neither curtailed nor increased his water consumption, so that the readings obtained under these conditions may be regarded as normal for the subject. Then on one or more days a measured amount of water was taken. Sometimes the amount was all drunk within a limited period. At other times, the drinking was spread over the day, half a litre or so being drunk at intervals. On the following days on which the



readings were continued, the usual amount of water was taken as in the preliminary control period. Each experiment therefore consists of three periods, which are indicated in the text as "pre-water," "water," and "post-water."

Fixed hours of taking the readings were adhered to. This was considered of importance, as there is evidence of a diurnal variation in blood-pressure. Subject 1 was on a constant weighed diet in each of the six experiments carried out on him. In the experiments on the other subjects, though the nature of the food and the time of taking meals were constant, the amounts of food taken were not weighed.

The influence of muscular exercise and emotional disturbances were as far as possible eliminated. All the subjects were on regular routine during the experimental periods. They were either engaged in laboratory work or were hospital patients. The readings were taken after the subject had been allowed to lie on a bed for fifteen minutes.

The instrument used was a Riva-Rocci with an Oliver screw compressor. The auditory method, whose accuracy has been demonstrated by MacWilliam, Melvin and Murray (1914), was adopted throughout.

#### EXPERIMENTAL DATA.

Experiments were conducted on (a) two healthy subjects with a normal blood-pressure, (b) two subjects with a pressure above normal, but with no evidence of a kidney lesion, and (c) fourteen pathological cases with a markedly raised pressure.

In (a) and (b) a total of fifteen experiments were conducted, each extending over several days with three or four readings per day. To reduce the rather extensive tabulated data the results are given in averages. In addition, one reading is given for each experiment on the last "pre-water" day, the last "water" day and the first "post-water" day. In (c) the data are given for four representative cases; the other cases are covered by a general statement. This abbreviated method of presenting the data, though it fails to give a full presentation of the details, enables all the essential features of the results to be brought out. The readings are in mm. mercury.

S = systolic pressure.

D = diastolic pressure.

P = pulse-rate per min.

PP = pulse-pressure, *i.e.* S minus D.

#### (a) *Healthy Subjects with Normal Pressure.*

Subject 1.—J. B. O—, aged 33 years in Experiment 1 and 38 in Experiments 2–6.

TABLE 1.—*Average of Six Experiments.*

	No. of readings.	S.	D.	PP.	P.
Pre-water .	33	116	66	50	76
Water .	23	110	64	46	72
Post-water .	26	106	61	45	72

TABLE 2.—9 a.m. Readings on last Pre-water, last Water and first Post-water Day of each Experiment.

No. of experiments.	1.		2.		3.		4.		5.		6.	
	S.	D.	S.	D.	S.	D.	S.	D.	S.	D.	S.	D.
Pre-water .	108	70	125	75	110	68	116	67	113	66	106	58
Water .	104	68	122	73	112	70	114	56	95	56	105	58
Post-water .	102	68	116	73	102	66	97	58	104	62	100	58

Five years intervened between Experiment 1 and Experiment 2. Experiments 2 to 6 were separated from each other by a period of two to four weeks. In all the experiments the subject was on a fixed diet.

Subject 2.—J. R. H—, aged 31 years.

TABLE 3.—Average of Three Experiments.

	No of readings.	S.	D.	PP.	P.
Pre-water .	16	113	69	44	58
Water .	11	110	69	41	58
Post-water .	13	106	64	42	52

TABLE 4.—Forenoon Readings in each Experiment on last Pre-water, Water, and Post-water Days.

No. of experiments.	1.		2.		3.	
	S.	D.	S.	D.	S.	D.
Pre-water .	112	72	116	73	112	68
Water .	102	66	115	72	111	70
Post-water .	110	68	113	70	106	61

An interval of about six weeks intervened between the experiments.

(b) *Subjects with High Blood-pressure, but no Evidence of Kidney Lesion.*

Subject 3.—J. I. M. I—, aged 21 years.

TABLE 5.—Averages of Experiment 1.

	No. of readings.	S.	D.	PP.	P.
Pre-water .	6	124	74	50	62
Water (3 lit. in one day) .	3	125	74	57	61
Post-water .	3	125	67	58	60

*Averages of Experiment 2.*

Pre-water .	6	121	68	53	62
Water (4 lit. in one day) .	2	120	69	51	66
Post-water .	3	118	65	53	60



*Averages of Experiment 3.*

Pre-water . . . . .	6	121	68	53	60
Water (two days), 4 lit. 1st day,					
2 lit. 2nd day . . . . .	4	120	61	59	55
1st post-water . . . . .	1	117	55	62	60
3, 4 and 5 post-water . . . . .	6	120	65	55	62

TABLE 6.—*Noon Readings on last Pre-water, last Water and first Post-water Day of each Experiment.*

No. of experiments.	1.		2.		3.	
	S.	D.	S.	D.	S.	D.
Pre-water . . . . .	126	76	125	69	122	71
Water . . . . .	134	84	115	74	116	64
Post-water . . . . .	123	69	117	65	117	65

Subject 4.—F. W. L—, aged 30 years.

TABLE 7.—*Average of Experiment 1.*

	No. of readings.	S.	D.	PP.	P.
Pre-water . . . . .	6	137	83	54	62
Water (3 lit. in one day) . . . . .	3	136	88	48	58
Post-water . . . . .	3	137	84	53	60

*Average of Experiment 2.*

Pre-water . . . . .	8	135	74	61	67
Water (three days), 1 lit. 1st day,					
3 lit. 2nd and 3rd days . . . . .	9	134	78	56	70
Post-water . . . . .	5	131	76	55	67

*Average of Experiment 3.*

Pre-water . . . . .	4	131	77	54	65
Water (6 lit. in one day) . . . . .	2	131	77	54	61
Post-water . . . . .	4	126	72	54	65

TABLE 8.—*Afternoon Readings on last Pre-water, last Water, and first Post-water Day of each Experiment.*

No. of experiments.	1.		2.		3.	
	S.	D.	S.	D.	S.	D.
Pre-water . . . . .	134	83	133	78	127	79
Water . . . . .	136	88	132	78	129	75
Post-water . . . . .	134	87	127	75	123	73

*(c) Pathological Cases.*

Subject 5.—G. D—, aged 29 years. Albumen constantly present in urine in traces. No other indications of disease.

TABLE 9.—*Readings at 12 o'clock noon.*

Date.					
3/3/20)	Pre-water	{	134	88	64
4/3/20)			134	88	61
5/3/20)	Water	{	130	85	69 2 lit. at 37° C.
6/3/20)			128	86	66 Ditto.
7/3/20)	Post-water	{	135	89	57
8/3/20)			133	88	64

Six other experiments showed a like result. On the day following the increased water intake the systolic pressure dropped 6 or 8 points and the diastolic 3 or 4. Both always returned to the former level on the second post-water day.

Subject 6.—James H—, aged 34 years; hospital patient. Case of parenchymatous nephritis.

TABLE 10.—*Readings at 2.15 p.m.*

Date.			S.	D.	P.	
10/5/20	Pre-water	.	180	113	62	
11/5/20)	Water	{	182	115	66	2 lit. at 37° C.
12/5/20)			164	108	78	Ditto.
13/5/20)	Post-water	{	172	112	61	
14/5/20)			156	101	74	
17/5/20	Pre-water	.	155	102	65	
18/5/20)	Water	{	153	102	63	3 lit. at 37° C.
19/5/20)			146	100	60	Ditto.
20/5/20)	Post-water	{	146	102	71	
21/5/20)			151	101	64	
22/5/20)			155	103	63	

Subject 7.—Jessie J—, aged 33 years; hospital patient. Albumen and granular casts in urine. This patient was given extra water on two successive days, and, after an interval, the increased water consumption was resumed and continued until the patient left hospital.

TABLE 11.—*Readings at 2.30 p.m.*

Date.			S.	D.	P.	
27/11/20	Pre-water	.	248	152	84	
28/11/20)	Water	{	248	156	84	1½ lit.
29/11/20)			234	140	95	3 lit.
30/11/20)	Post-water	{	206	136	90	
1/12/20)			231	153	89	
2/12/20)			244	153	84	
3/12/20	Pre-water	.	234	136	86	
4/12/20)	Water	{	211	140	95	2-3 lit. daily;
13/12/20)			198	130	84	amount not
23/12/20)			206	130	92	measured.
1/1/21)			205	134	90	



Subject 8.—Miss C—. Chronic Ménière's disease. Increased water ingestion continued from 15/5/21 as treatment.

TABLE 12.

Date.		S.	D.	P.	
15/5/21	Pre-water	212	125	86	
22/5/21	Water	174	105	80	2-3 lit. per day ; amount not measured.
29/5/21		170	105	80	
24/7/21		165	102	80	

In all the other pathological cases a decrease in arterial tension, something similar to that in the cases recorded, was obtained on the day following increased water intake. The decrease was usually accompanied by an alleviation of subjective symptoms, such as headaches, insomnia, giddiness, where these were present.

#### DISCUSSION OF RESULTS.

##### *Water Days.*

The two normal subjects show little or no alteration of pressure on the days on which the extra water was drunk. Any change is towards a decrease rather than an increase. In the two subjects with high pressure, however, there is in the first experiments a marked increase in pressure, which is more marked in the diastolic than in the systolic readings. This is doubtless due to increased volume of blood.

In the two cases with high blood-pressure it is probable that the renal efficiency was impaired and the diuresis was less effective than in the other two healthy subjects in keeping pace with the absorption of water from the intestine. The resulting increase in the volume of the blood would cause a rise in the systemic pressure which would be most marked in the diastolic readings. Miller and Williams (1921) have shown that in patients with hypertension large amounts of fluid may cause a very decided increase in blood-pressure. They gave in one day as much as 10 litres of water, which was introduced by a Rehfuß tube. It had already been noted by one of us (Orr, 1920) that in cases of relatively high blood-pressure an increased pressure may occur on the consumption of large quantities of water.

In the pathological cases little or no increase in the pressure is shown on the water days, because in these cases care was taken by noting the output of urine to ensure that the water was not being administered more rapidly than the kidneys could excrete it.

##### *Post-water Days.*

*Blood-pressure.*—In all the subjects there is a definite fall of both systolic and diastolic pressure on the day following the increased intake of water. In some cases, as in Subject 5, the pressure returned to the pre-water level on

the second post-water day. In most of the cases, however, the pressure, and more especially the diastolic pressure, tended to remain down for more than one day.

*Cumulative effect.*—There is some evidence of a cumulative effect in Tables 2, 4, 6 and 8 where corresponding readings in successive experiments on the same subject are compared. An interval of two to four weeks intervened between the experiments. In each experiment the readings tend to be lower and the fall on the post-water day to be greater than in the preceding one. Further, the rise on the water day, which is quite definite in the first experiment in Tables 6 and 8 (cases of high blood-pressure), is either slight or absent in the subsequent ones. In striking contrast to this cumulative effect in these subjects is the case of Subject 5 (Table 9), where repeated attempts failed to produce any results beyond the first post-water day. Reasons are adduced later for believing that the fall in pressure is due to the influence of the water on the metabolism of protein. The difference in the cases is probably due to the fact that Subjects 1, 2, 3 and 4 were on an ordinary diet, while Subject 5 was on a very low protein diet as a therapeutic measure.

*Pulse-pressure and pulse-rate.*—In Subjects 3 and 4 (high blood-pressure with no detected kidney lesion) the influence of the increased water intake on pulse-pressure is variable. A definite fall in all the normal and pathological subjects is shown in the following table:

TABLE 13.—*Pulse-pressure.*

	Pre-water day.	First post- water.	
Subject 1 . . .	47	45	Average of all experiments.
Subject 2 . . .	44	42	" " "
Subject 5 . . .	46	42	" " "
Subject 6 . . .	67	60	First experiment.
	51	44	Second experiment.
Subject 7 . . .	96	70	
Subject 8 . . .	97	65	

In the pathological subjects there is a tendency for the pulse-rate to rise on the water days. On the post-water days it falls to or below the level of the pre-water days. In all the other subjects the pulse-rate is slower after the ingestion of the water.

A consideration of the pressure and pulse-rate shows that the fall in the systemic pressure produced by the passage of water through the system is not counter-balanced by an accelerated pulse-rate or a more powerful heart-beat. The regulatory nerves of the circulatory system are normally efficient in preventing a fall in blood-pressure below the required level. The fact that diminished pressure following water ingestion is not accompanied by increased rate or force of the heart-beat seems to indicate that the lower level established on the "post-water" days does not embarrass the organism and necessitate the calling into action of the regulating nervous mechanism. If this view be



correct the "post-water" systemic pressure is nearer the optimum than the pre-water, and the effect of the increased water ingestion has been to remove a cause of an unnecessarily augmented blood-pressure.

#### COMPARISON OF RESULTS WITH PREVIOUS WORK.

Hay (1882) by strong saline cathartics removed water from the blood and found, "contrary to expectations," a marked rise in blood-pressure, as shown by sphygmographic tracings. He suggested that the salts used—sodium sulphate and magnesium sulphate—had stimulated the tunica intima of the vessels and induced a contraction of the smaller arteries and capillaries. MacWilliam, Mackie and Murray (1904), however, found that an immediate fall of blood-pressure is produced by the introduction of sodium sulphate into the blood-stream and no change by magnesium sulphate. There is evidently no contraction of arterioles due to irritation by the salts, so that the explanation suggested by Hay seems invalid. In the light of the results presented here, the more probable explanation is that the reduction of the volume of the blood, by the abstraction of water produced by the cathartics, reduced diuresis and the flow of fluid through the tissues—a condition opposite to that produced in these present experiments, and followed by an opposite result. The present results are therefore really in agreement with and are an extension of Hay's original observations.

Benedict and Carpenter (1918), in the course of an inquiry into the influence of water-drinking on the energy exchange, took a few readings of systolic blood-pressure on four subjects before and after the ingestion of 500 c.c. water. They concluded that no significant change in pressure was produced by the ingestion of water. The average readings obtained by them for the first three subjects were: Before water, 105, 113 and 120; after water, 102, 103 and 107.

In the fourth subject the readings were: Before water, 117 and 116; after water, 126, 128, 119 and 117.

If the readings were taken on the water day, as was evidently the case, the records are confirmatory of the results presented here. In three cases there is a fall on the water day as occurs in our normal subjects. In one case there is an immediate rise in pressure, which evidently disappears as the extra fluid is eliminated. A reading of the fourth case on the post-water day would have been of interest. It is unfortunate that the records are so scanty and lack any reference to diastolic pressure.

Miller and Williams (1912), working with three patients with very high blood-pressure, found that the introduction of 10 litres of water in one day markedly raised the systemic pressure on the day on which the water was given. Fortunately, in two of the cases, readings were taken on the following day, and recorded in the protocols, though no reference is made to these in the text. They are as follows:

#### *Protocol 1.*

		S.		D.		PP.		Body wt. lbs.
20/12/19	. 9 a.m., before water	. 160	.	108	.	52	.	124.2
21/11/19	. 9 a.m., after water	. 140	.	90	.	50	.	125.8

*Protocol 2.*

Water was given during the night of December 26th and 27th.

		S.	D.	PP.	Body wt. lbs.
26/12/19	6 p.m., before water	170	100	70	131.7
27/12/19	3 p.m., after water	140	90	50	133.0
28/12/19	11 a.m., after water	155	90	65	?

Even though the body-weight seems to indicate that the volume of the blood had not returned to normal, a marked decrease in the systemic pressure and in the pulse pressure in the post-water period, is evident.

THE CAUSE OF THE FALL IN BLOOD-PRESSURE AFTER INCREASED  
WATER INGESTION.

In recent years there has been an accumulation of experimental evidence on the influence of certain nitrogenous decomposition products in raising blood-pressure. Dixon and Taylor (1907) found in extract of placental tissue an active principle that caused a rise in blood-pressure due to contraction of arterioles. Rosenheim (1909) showed that the pressor effect was due to *p*-hydroxyphenylethylamine. About the same time Barger and Dale (1909) showed that this substance was the chief body concerned in the pressor effect that could be got from an extract of putrid meat. It is derived from tyrosine by anaërobic disintegration. Several other amines which exert a pressor influence have been described by Barger and Dale (1910). It has been shown by Dale and Dixon (1909) that some at least of these amines can be absorbed from the alimentary canal and produce pressor effects when so administered. The possibility of these pressor substances arising by means other than bacterial action has been shown by Emerson (1902), who found a pressor substance in the autolysis of pancreas under conditions that excluded putrefaction, and by Langstein (1902), who obtained the same body in prolonged peptic digestion of egg albumen. The results of these investigations show that certain amines which exert a pressor influence may originate in the intestine and be absorbed to the blood-stream. They also suggest the possibility of certain nitrogenous substances with a pressor influence arising in the tissues as a result of perverted or incomplete metabolism of protein.

A great amount of work has been done on the influence of water-drinking on protein metabolism. A review of the older literature is given by von Noorden (1907). There is general agreement that the drinking of water produces a rise in the excretion of nitrogen in the urine on the first day of increased water consumption. There is, however, diversity of opinion as to whether this represents merely a flushing out of end-products or an increased catabolism of protein. Fowler and Hawk (1910) found that increased water consumption was accompanied by more rapid digestion and more complete absorption of the protein of the food, and also by a decrease in faecal nitrogen and in the bacteria of the faeces. One of us (Orr, 1914), working on the influence of increased water consumption on the metabolism of protein in the tissues, obtained results that suggested that an increase in the amount of water passing through the system causes an acceleration of both the catabolic and



the synthetic phases of protein metabolism, resulting in the one case in the production of innocuous final products, chiefly urea, which are excreted, and in the other in completely synthesised protein. The results showed that the stimulus to protein metabolism may continue for two or three days after the extra water has passed through the system.

A consideration of the results of work on the influence of increased water intake on the protein metabolism suggests that at least three factors may be involved in the reduction of blood-pressure noted in the experiments recorded here.

The initial flushing-out process, as evidenced by the increased excretion of nitrogen in the urine, may remove pressor substances from the system.

The results obtained by Fowler and Hawk (1910) suggest that anaërobic disintegration of nitrogenous material in the large intestine may be diminished, with a consequent reduction in the formation and absorption of pressor substances.

It is possible that substances that produce arterial contraction arise in cases of sluggish or perverted metabolism or under conditions of protein surfeit. The acceleration of the metabolism of protein with the more rapid formation of innocuous final products would lead to the elimination of these pressor substances.

*Note.*—We wish to express our indebtedness to Prof. McWilliam, F.R.S., for the kindly interest taken in the work recorded here, and for valuable advice and guidance rendered from time to time during the course of the investigation. We are indebted also to Drs. T. Fraser, F. K. Smith and W. R. Pirie for facilities for making observations in wards in the Royal Infirmary, Aberdeen, on the pathological cases.

#### CONCLUSIONS.

(1) Both in apparently normal subjects and in pathological cases with high arterial tension copious ingestion of water is followed by a decrease in blood-pressure.

(2) It is suggested that the fall in pressure is due to the elimination of pressor substances that cause arterial constriction.

(3) When diuresis is unable to keep pace with the intake of water, as may occur in renal inefficiency, the fall in pressure is preceded by a rise above the original level.

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# DETAILS OF THE TECHNIQUE ADOPTED IN FOLLOWING WEIGL'S PLAN OF FEEDING LICE INFECTED WITH THE VIRUS OF TYPHUS FEVER BY RECTAL INJECTION.

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THE author had the advantage, when in Warsaw in the spring of 1920, of seeing Dr. Weigl demonstrate his plan of feeding lice by inserting a minute capillary pipette into the rectal opening of the insect and injecting a meal of blood. Dr. Weigl did not on this occasion demonstrate any attendant details of the sterilisation of the insect or the pipette prior to the operation, nor is any mention made of the necessity for such precautions in the summary in English of his paper (1920).

The alimentary tract of *Pediculus humanus*, and, so far as microscopic evidence goes, that of some species of lice occurring on animals other than man, is normally sterile; and experience shows that in the case of *Pediculus humanus* bacterial contamination of the gut is frequently, if not always, followed by the speedy death of the insect.

When breeding lice in gauze-covered boxes, evidence is obtained which suggests that bacterial contamination of the gut is one of the normal causes of death among captive lice, and that the death-rate from this cause is greatly accentuated by moist or very humid conditions, etc., in the boxes. It is therefore necessary, when feeding by rectal injection, to adopt measures to reduce the danger of infection, when inserting the pipette, to a minimum.

Sikora (1920) gives an account of the method she adopted in sterilising the lice she used for injection experiments (immersion in 1/1000 mercuric chloride followed by washing in iodine and hyposulphite of soda), but gives no indication as to whether the solutions were in diluted alcohol or water. It may be mentioned in this connection that lice can survive ten minutes' immersion in 85 per cent. alcohol. I have tried the method indicated by Sikora using 85 per cent. alcohol as the solvent, but personally have succeeded better by immersing the insects for from two to four minutes in 2 per cent. "lysol" (temperature about 60°–65° F.).

The lice\* are transferred from the "lysol" to sterile water and removed thence to filter-paper placed in Petri dishes to recover. Activity is regained more rapidly by incubation at 90° F. for a few minutes.

\* It was pointed out by Weigl that it is much easier to inject females than males; the death-rate from faulty technique speedily testified to the correctness of this warning.

To inject the infecting material or subsequent food (I have found whipped human blood most satisfactory), the louse is held in position under a slip of paper on a glass slide placed on the stage of a binocular dissecting microscope; the anal extremity of the insect should project a little way beyond the paper to allow of a clear view of the passage of the pipette. The magnification required is about sixteen diameters.

A finely drawn capillary pipette is redrawn in a minute flame and the tapering point cut off as nearly as possible at right angles by the pressure of a triangular pointed needle on a glass slide. One soon learns to judge the necessary external diameter (about 0.1 mm.) by comparison with the tapering point of the needle. The cut end of the tube must be smoothed by flaming, and this item of the technique I have found to require considerably longer practice than the correct insertion of the pipette into the rectal passage, which lies close beneath the dorsal skin. After loading, the pipette is inserted a short distance (a little beyond the last segmental incision) up the rectal passage and sufficient fluid is forced into the stomach by gradual pressure on a well-fitted rubber teat. Weigl used, and showed in his illustration, a small injection syringe, but in my experience a good teat is quite powerful enough.

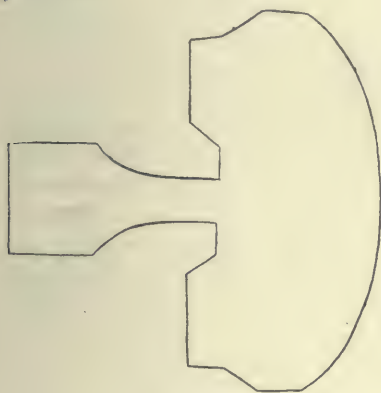


FIG. 1.

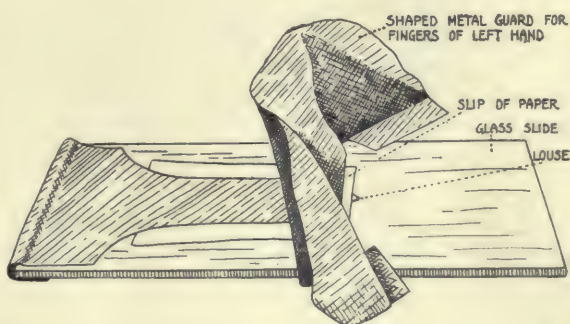


FIG. 2.

Until one has had considerable practice there is some danger, in working with infecting material, of pricking a finger before the pipette comes into the field of the microscope. A metal guard covering the fingers may be cut out of any thin soft metal (Fig. 1) and bent into shape as required (Fig. 2).

This safety device renders the operation somewhat slower, and is, of course, quite unnecessary when feeding with sterile blood.

The pipette is washed out after each injection with distilled water or salt solution, and then sterilised in boiling water before taking up the blood or infecting material for the next operation.

To Major Patton, I.M.S., I am indebted for the suggestion to touch the skin and hairs surrounding the rectal opening with a minute droplet of 85 per cent. alcohol immediately prior to the insertion of the pipette.



Two meals per day are necessary if the lice are to be incubated at 90° F.; one meal is sufficient at 80° F., and lice may be kept over the week-end for forty to fifty hours on a single meal if kept at 65° F. Lice have been successfully kept alive by this system of feeding for twenty-seven days, and are sufficiently well nourished to develop eggs and lay them if the temperature is high enough.

Healthy uninfected lice are generally easier to feed than those which are heavily infected with *Rickettsia prowazeki*. They further appear to be better nourished by their meals, and less liable to have the lower intestine obstructed by solid particles of excreta than the infected lice.

In view of the success which attends rectal feeding, the question, so frequently debated, of the use of salivary fluid is again raised. Presumably, owing to the extreme specialisation of the piercing and sucking apparatus, and the fact that the salivary ducts enter the base of the pocket beneath the palate, the salivary fluid does not obtain access to blood rectally injected. Its service, if any, as an aid to the digestion of the blood would seem, therefore, to be easily dispensed with, although, of course, its suggested use in connection with the prevention of coagulation is not called in question when the food given is defibrinated blood.

Lice which have been well fed rectally appear to have ravenous appetites when allowed to feed naturally, even on a monkey, but it is not perhaps safe to infer from this that rectal feeding does not assuage their appetites.

It was very noticeable during the course of experiments how short a time human lice survived when fed naturally on a *Macacus* monkey in comparison with those fed *per rectum* on whipped human blood.

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A communication dealing with results of intra-rectal infection of lice with guinea-pig platelet emulsions containing typhus virus will appear in the next number of this Journal.

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## THE POISONOUS PROPERTIES OF COLLOIDAL SILICA. I: THE EFFECTS OF THE PARENTERAL ADMINISTRATION OF LARGE DOSES.

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THE starting-point of the investigation described in the present series of papers was an observation made in 1918 by one of the authors (W. E. G.) when working at the subject of anaërobic infections, namely, that intraperitoneal injections of small doses of freshly prepared colloidal silica in mice are lethal.

During the last twenty years a considerable number of medical memoirs on silica have been published; for the most part they are of chemical and therapeutic interest. The majority of the chemical investigations have been merely the mechanical estimation of the percentage of silica in the organs, tissues and fluids of the animal body. The figures published by different observers show great discrepancies, but there is general agreement that silica is a frequent—and possibly a constant—constituent of most of the tissues of the body. Schulz (1903) concludes from the results of a large number of analyses that silica is an essential constituent of connective tissue, and that in young animals the proportion of silica present is greater than in older animals. Kahle's (1921) figures for the analysis of the pancreas do not support this view however. Schulz, Kühn and other investigators believe that silica is metabolised in the body.

In Germany silicic acid, in one form or another, is used in the treatment of pulmonary tuberculosis (Kobert, Kühn (1921), and others). The substance is administered by mouth or by intramuscular injection. The basis of the treatment appears to be, first, that almost immediately following an injection of a small dose there is a considerable increase in the number of leucocytes in the peripheral blood; and secondly, that silica stimulates the production of connective tissue—presumably specifically around the tubercular lesions. A critical examination of the treatment has been made by Roth (1921), whose paper contains a long list of references.

Sodium silicate has been employed by Scheffler, Sartory and Pellissier (1920) and by Kühn (1921) in the treatment of arteriosclerosis.

In spite of the stimulus which the use of a drug in practical medicine gives to laboratory investigations, there has been no systematic and thorough study of the pathological effects which follow the administration of colloidal silica.



Friedenthal (1900), in a footnote to a paper on the passage of colloids through the intestinal wall, states that silicic acid when injected intravenously may kill an animal; he attributes death to the formation of insoluble precipitates of calcium and silica. Kobert utters a warning against the too rapid injection of silicic acid into the blood-stream, pointing out the possibility of the occurrence of embolism. Zuckmayer (1921) quotes an experiment of Siegfried in which a cat died 40 hours after being injected intravenously with 81 mgrm. of silica sol—20 mgrm. per kilo body-weight.

The same author has studied the effects of administering small doses parenterally in dogs. His investigations were stimulated and limited by the consideration of silica as a drug, and give very little information.

Before describing the results obtained by injecting silica sol by different routes, a brief account of the elementary chemistry of silica will be given.

#### CHEMISTRY OF SILICA.

Silica ( $\text{SiO}_2$ ) occurs abundantly in nature in an amorphous form, and also in a crystalline condition. It is insoluble in water and in all acids except hydrofluoric acid; it can be dissolved by alkalis, *e. g.* by boiling the amorphous powder in a solution of sodium carbonate, to form alkaline silicates, which are easily decomposed by weak acids.

Silica may be obtained in the colloidal state either as a sol or a gel, the form depending upon the extent of hydration of the silica molecule. The sol form, usually known as "orthosilicic acid," to which the formula  $\text{Si}(\text{OH})_4$  is assigned, may be filtered through a Berkfeld candle, and can be sterilised by boiling or in the autoclave. The gel form, "metasilicic acid," with the formula  $\text{H}_2\text{SiO}_3$ , can be prepared directly by the action of acids on alkaline silicates, or may be obtained by allowing the sol form to gel—a process which is irreversible; that is to say, it is not possible to obtain afresh a solution of colloidal silica from one which has been allowed to gel.

The description of these colloids as acids is not justified by the properties of the substances themselves; it is convenient to regard them as acids in order to provide a satisfactory classification of the mineral silicates. Strictly speaking, however, it is probably an error to put them in the same list as the weak acids (Le Chatelier, 1914). They are practically neutral to litmus, and have a faint acrid taste which persists.

The circulation of silica in nature depends upon the formation and ready decomposition of hydrated silica and of complex-balanced solutions of the alkaline silicates.

When used in this and the following papers the terms "soluble silica" and "silica sol" will refer to orthosilicic acid, and the term "silica gel" will refer to metasilicic acid. The strength of the respective substances will be given as the weight of silica ( $\text{SiO}_2$ ) contained in 100 c.c. of the sol and in 100 grm. of the gel. The silica sol used throughout this investigation was prepared by adding a dilute solution of sodium silicate—"waterglass"—to an excess of strong  $\text{HCl}$ , and then freeing the mixture of the  $\text{HCl}$  and  $\text{NaCl}$  by dialysis. The water-clear sol was then filtered through a Berkfeld candle and stored in test-tubes.

## THE EFFECT OF THE INJECTION OF LARGE DOSES OF SOLUBLE SILICA.

*Subcutaneous Injections.*

When 0.1 c.c. of a 1 per cent. solution of orthosilicic acid is injected under the skin of a mouse an inflammatory reaction occurs (Bullock and Cramer, 1919); the blood-vessels at the site of the injection become dilated, fluid is poured out, large numbers of white cells and a few red blood-corpuscles escape into the œdematous area, which is to the naked eye a flat, colourless, gelatinous swelling from one-half to one square centimetre in size.

*Intraperitoneal Injections.*

The effects obtained by intraperitoneal injections vary with the degree of dispersion of the silica sol. The freshly-prepared sol is much more toxic than one which has been kept for a long time, *e.g.* three months. The sol always tends to gel; first the viscosity increases, and then, with the lapse of time, definite gelation begins, and proceeds rapidly until all the sol is converted into gel. These changes may be retarded by means of traces of acid or alkali or by a protective colloid such as albumin, but they cannot be prevented. Coincident with this the toxicity in intraperitoneal injections decreases, whilst for intravenous injections the reverse is the case, as will be shown later. It is evident, therefore, that any quantitative statement of poisonous doses must be qualified by a description of the state of the sol. With regard to intraperitoneal injections it may be stated (1) that doses of 1 to 2 mgrm. of freshly-prepared sol kill a mouse, and that 30 mgrm. kill a guinea-pig of 250 grm. weight; (2) that the sol which has been allowed to become opalescent and whose mobility has diminished kills a mouse in doses of 5 mgrm. and a guinea-pig in doses of 50–60 mgrm.; and (3) that the gel does not kill in doses which it is practicable to administer.

There is another difference in action between the highly-dispersed sol and that which has stood for some time. The effects of the former are shown within a few minutes after injection, while the latter produces no symptoms within 12 hours. These points are illustrated in the following protocols.

*Experiment 20 G.*—October 10th, 1921: A sample of silica sol, which had been dialysed against distilled water until the chlorides had been removed, was filtered through a Berkfeld candle, and immediately injected intraperitoneally into mice in the doses indicated below. Subsequent analyses of the filtered sol showed the concentrations to be 1 per cent.

Mouse No.	Dose injected.	24 hrs. after.	48 hrs. after.
1	0.1 c.c.	†	—
2	0.1 c.c.	Very ill	†
3	0.1 c.c.	Very ill	Dying. Killed at 52 hrs.
4	0.2 c.c.	Very ill	†
5	0.2 c.c.	†	—
6	0.2 c.c.	Very ill	†

Within five minutes of the injection all the animals became quiet and reluctant to move; later they began to shake and the legs became unsteady. After an hour the mice recovered and then appeared to be quite well.



*Post-mortem* there was nothing of special importance to note beyond a dilatation of the intestines, the peritoneal surface of which showed here and there fragments of fibrin. Films of the peritoneal exudate contained a large number of lymphocytes, polymorphonuclear leucocytes, endothelial cells and a few red blood-corpuscles. In some of the animals a fair number of bacteria—chiefly a Gram-negative bacillus—were present, in others few or none.

*Experiment 21 G.*—October 10th, 1921: Six mice were injected intraperitoneally, each with 0.3 c.c. of 1 per cent. silica sol which had been kept for 3 months; the sol was faintly opalescent. The animals remained active and well after the injections. Twenty-four hours afterwards all six mice were ill; 48 hours after the injection 5 had recovered, 1 was dead.

Guinea-pigs are affected in the same way after the injection of larger doses—from 3 to 5 c.c. of 1 per cent. sol, made isotonic with salt immediately before use. When the sol is freshly prepared the injected animals become ill within a few minutes; they often display a curious rhythmical jerking of the head which may persist for half an hour. The legs are spread out in a helpless fashion, the respirations become rapid, and the animals appear moribund. When the animals are disturbed, however, they move away, thus indicating that the distress is temporary. In 30 to 40 minutes they recover and afterwards appear to be quite well. Death may take place in 24 hours or may be delayed for several days.

We are not yet able to state definitely the cause of death. At first we were inclined to attribute it to a septic peritonitis, but this explanation must be rejected. Whilst in a fair proportion of the animals—and especially mice, which are prone to infection—bacteria are found *post-mortem* in large numbers in the peritoneal exudate, in many the numbers found are few or none. In guinea-pigs which die 4 to 7 days after injection the intestines are matted together by fibrinous material in which no organisms are found; the liver and kidneys are pale, and show, when examined microscopically, degenerative changes.

#### *Intravenous Injections.*

The results which follow the intravenous injection of silica sol vary with the method of administration, whether, for example, the total dose is administered at once or in fractions at shorter or longer intervals.

An animal may be killed in a few minutes by a single large dose which, for the same sample of sol, is fairly constant, body-weight for body-weight, for rabbits, rats, guinea-pigs and mice. With the freshly prepared sol the lethal dose is roughly 100 mgrm. per kilo body-weight. When the sol has been kept for a long time and gelation has begun the killing dose is less, a mechanical factor probably being added to the toxic. Also when the concentration is high, *e. g.* 3 per cent., the sol is more effective than when the concentration is low. This probably is only another way of stating that gelation reduces the killing dose, since the higher the concentration the more rapidly the sol gels. The cause of death in these cases is intravascular clotting.

When a single lethal dose is administered in fractions at short intervals, *e. g.* every half hour, the animal remains quite well until the last two or three portions are injected. After the last fraction it succumbs.

If the sol be given in large but sublethal doses—40 to 60 mgrm. for a rabbit—at daily intervals, the animal dies after 3 or 4 days, and under these conditions death is due to profound degenerative changes of the liver, kidneys and other organs, together with hæmorrhages in the intestinal mucosa, in the tracheal mucous membrane, in heart-muscle and elsewhere. It is evident that these cases may be separated naturally from those in which death takes place almost immediately after the completion of the injection. We shall, therefore, divide this section into two parts, the first (A) dealing with those cases where death occurs rapidly, and the second (B) with those in which death occurs after several days.

A. *Rapid death*.—The results obtained may be best described by reproducing protocols of experiments.

*Experiment 29*.—June 21st, 1921: A healthy mouse was injected intravenously with 0·3 c.c. of 1 per cent. silica sol. The solution was injected very slowly. The mouse appeared to be perfectly well when the injection was completed and walked about normally. Two minutes afterwards the animal became unstable on its hind legs, which shook when it attempted to walk; the weakness spread rapidly to the fore limbs, the animal became convulsed and died. The mouse was dead within three minutes from the time of completion of the injection.

A *post-mortem* examination was made at once. The right side of the heart and the large venous trunks were distended with clotted blood; the left ventricle was empty.

The lethal dose varies from 1 to 3 mgrm. When the solution of silica has begun to gel, as is shown by the development of a faint opalescence, the mouse may be killed by a dose of 1 mgrm.; 2 mgrm. of a freshly-prepared solution is usually supported by a mouse of 20 gm. weight. When death occurs immediately it is due to intravascular clotting. Sometimes, and especially after the injection of a dose bordering on the lethal, *e. g.* 2 mgrm. of freshly-prepared solution, an animal may become ill and die in from 2 to 24 hours. The cause of death in these cases is not always easy to determine. The only lesion which we have found microscopically is a necrosis of the convoluted tubules in the kidney.

The next experiment illustrates the effect of a dose which was barely lethal.

*Experiment 12 G*.—March 14th, 1921: Six mice were injected intravenously, each with 2 mgrm. (in 0·2 c.c. of saline) of soluble silica. On the morning of March 15th two were found dead and were thrown away. One mouse was very ill and was killed with chloroform. *Post-mortem* there was an unusual redness of the subcutaneous tissues; the liver was pale, the gall-bladder distended with dark bile; the intestines were pale, contracted and still; the kidneys were pale, and the bladder was distended. The heart and lungs were normal. Microscopical examination revealed tubular necrosis of the kidney. A fourth mouse was killed on March 17th; this animal also showed necrosis of kidney tubules. The fifth animal died April 9th, 1921, and was discarded.

The last mouse of this series was killed on April 28th, 1921. The kidneys were small and contracted. Microscopic examination showed the replace-



ment by connective tissue of tubules which had been necrosed and had disappeared.

*Experiment 28.*—June 22nd, 1921 : A healthy rabbit weighing 1275 grm. was injected intravenously with a clear 1 per cent. solution of silica, to which was added immediately before use a sufficient volume of saturated salt solution to make the resultant mixture isotonic with blood. Ten cubic centimetres were injected very slowly ; the rabbit appearing quite well, a further 9 c.c. was injected. Whilst the last few c.c. were being delivered the animal became rather restless and uneasy. The total dose administered was 190 mgrm. Immediately the injection was completed the rabbit was put on the floor and observed. It began to walk about, but had difficulty with its hind legs ; it then lay quiet, then kicked and struggled, rolled over and died. The time which elapsed between completion of the injection and death was 3 minutes.

A *post-mortem* examination was made immediately. The subcutaneous tissues were pallid and no bleeding occurred when skin was cut away. The liver was purple ; active peristalsis of the intestines was observed. The vena cava was distended. As the left kidney was cut away a clot came from the renal vein. The lungs were collapsed and firm on section. The right side of the heart was dilated and full of blood-clot, the left side was empty. The pulmonary vessels and the inferior vena cava were filled with clot.

*Experiment 35.*—June 30th, 1921 : A healthy rabbit weighing 1700 grm. was injected intravenously with 2 c.c. of a 3 per cent. silica sol, which was opalescent and less mobile than water. The solution was injected slowly, the injection taking half a minute. The rabbit was restless during the process, and when put on the floor, after the injection was completed, staggered, struggled and died. Death took place two minutes after the beginning of the injection.

The right side of the heart was found to be dilated and filled with blood-clot, the left side almost empty. In this case the dose injected was 60 mgrm.

*Experiment 43.*—August 14th, 1921 : A healthy rabbit weighing 1300 grm. was injected intravenously with 1 c.c. soluble silica of a strength of 1.4 per cent. at 8.35 a.m. and again at 9.10 a.m., 9.50, 10.20, 11.5, 11.40 and 12.20 p.m. The total amount injected was, therefore, 7 c.c., which represented 98 mgrm. of silica.

After the fourth injection the respiration-rate increased. At 11.43 a.m., after the sixth injection, the rabbit was disinclined to move and was panting. At 12.6 p.m. the animal tried to rise, but did not succeed. After the last injection, 12.20 p.m., the animal struggled, took single deep inspirations, rolled on its side and died.

The carcase was examined immediately. The heart was still attempting to beat, the right side was engorged. There was no clot in the right ventricle or inferior vena cava. The blood was dark brown in colour throughout the body.

In a subsequent experiment of the same kind we have found that hæmolysis of the blood occurs, and that the bladder contains urine stained with hæmoglobin.

This and other similar experiments have shown that silica sol when injected in comparatively small doses at short intervals either accumulates

within the blood—that is to say, that excretion is slow—or that lesions that may be caused by small doses are summated. The immediate cause of death in these cases we have not yet elucidated; it is not, however, due to universal clotting. Clot-formation in the smaller vessels we have not excluded.

Our colleague, Dr. Lovatt Evans, very kindly estimated the effect of 100 mgrm. of colloidal silica on the basal metabolism of a full-grown rabbit. The effect was *nil*. It may be concluded, therefore, that a single large dose does not affect, immediately, the metabolic activity of the tissues.

The clotting of the blood which has been described was at first attributed to a direct action of the silica on the blood. This view is easily understood when it is realised how readily a solution of silica gels in the presence of electrolytes. The addition of 2 c.c. of 4 per cent. silica sol to 4 c.c. of broth leads to the formation, in an hour, of a fairly stiff gel, which, when sloped, is suitable as a solid culture medium for bacteria. It is easy to imagine that the colloidal silica injected directly into the blood-stream becomes a gel and that clotting begins round the particles of gel, gradually spreading through the vascular system. This conception is supported by the fact that the sol which has begun to gel acts in smaller doses than the pure sol. When colloidal silica is added to blood *in vitro*, however, the rate of clotting is not accelerated by doses which proportionately are certainly lethal to the animal supplying the blood. The only noticeable effect on the blood is a slight hæmolysis of the red cells. This, of course, was to be expected, since it is known that silicic acid and lecithin together are able to lacerate red blood-corpuscles.

The rapid production of clot in the blood-vessels of the living animal must, it follows, be due to an action on some other tissue than the blood, and most probably on the vascular endothelium.

The following experiment, one of several, was designed to test this point. It was carried out by Capt. S. R. Douglas (using Wright's method), to whom we desire to express our thanks.

A healthy rabbit, weighing 2·5 kilos, was bled, and the clotting time of the blood taken; this was found to be 6 minutes. The test was repeated in 10 minutes, and the time again found to be 6 minutes. The rabbit was then injected with 50 mgrm. of colloidal silica, made isotonic with blood.

The following observations were then made:

A. 10 minutes afterwards blood was taken: it was very dark in colour; its clotting time was 2 min. 45 sec.

B. 30 minutes afterwards the blood clotted in 2 minutes.

C. 4 hours afterwards the blood clotted in  $3\frac{1}{4}$  minutes.

D. 24 hours afterwards the blood clotted in 8 minutes.

In another experiment, in which the same amount of soluble silica was injected, the clotting time fell from 4 minutes to  $\frac{1}{4}$  minute in 2 hours.

It will be noticed from these experiments that the capacity to clot is rapidly augmented, and that 24 hours later it is diminished. It seems to us impossible to resist the conclusion, which to some extent is supported by the effects of intraperitoneal injections, that colloidal silica acts upon the vascular endothelium.

B. *Slow death*.—In this part of our investigation we have done but few experiments, and the total number are, therefore, given in the following table:



	Wt. of rabbit.	Dose.	No. of doses given.	Result.
1	2100 grm.	24 mgrm.	13	Animal quiet and off its food during the first ten days; tending to recover after 10th day; killed 14th day.
2	1700 „	24 „	4	+ 4 days.
3	1100 „	36 „	4	+ 4 „
4	4400 „	36 „	2	+ 3 „
5	1050 „	60 „	3	+ 3 „
6	2050 „	60 „	4	+ 4 „
7	3000 „	60 „	2	+ 4 „
8	2850 „	60 „	2	+ 2 „
9	2400 „	72 „	3	+ 3 „
10	3850 „	72 „	2	Ill for several days; recovered; killed 14th day.

Thus, of the ten animals used, one alone survived repeated intravenous injections; one, which was injected twice with the largest dose given in the series of experiments, became ill, but was allowed to recover, and was killed later. All the rest died on the third or fourth day. An analysis of the protocols of the separate animals shows that, although there are slight differences clinically in the course of the illness produced—one, for example, showed a conjunctival hæmorrhage, the others did not—and in the *post-mortem* and microscopical findings, the differences are not sufficiently great to warrant a separate description for each animal. A general account of the experiments will be given which is fairly applicable to all.

After the first injection if the dose is 36 mgrm. or more, but after the second injection when the dose is less, the rabbit becomes quiet and listless and loses its appetite. Its weight falls a hundred or even three to four hundred grammes, due probably to the emptying of the intestines and to loss of fluid which is not replaced. There is no regular effect on the temperature of the animal. The condition of debility and lassitude passes on to a pronounced weakness, and the animal dies without exhibiting any premonitory sign that death is imminent. The urine, which at the beginning of the experiment and during the first day contains no albumin, contains, on the third (or fourth) day, an amount which gives a thick cloud on boiling and acidifying. Urine taken from the bladder *post-mortem* contains an enormous number of casts and a few epithelial and red blood-cells.

The most prominent features at *post-mortem* examination are the occurrence of petechial hæmorrhages—in the skin, in the mucosa of the stomach and intestines, the lungs, the thymus, heart muscle and elsewhere—and the evident degeneration of the liver, which is pale and shows a finely mottled surface. The spleen is always enlarged, though to a variable extent, the edges being rounded and the natural depressions and rough markings lost in the general swelling of the organ; the colour varies from a salmon colour to a deep red. The kidney is swollen and pale; on section the larger vessels stand out as deep red points. The lungs are hæmorrhagic. In two animals of the series the ureters showed as red cords, which was found to be due to

hæmorrhages in the submucosa and in the muscular coat. The adrenals are soft and redder than normal; the pancreas, thymus and thyroid are not conspicuously altered. Lymphatic glands, subcutaneous, abdominal and thoracic, are generally swollen and soft and sometimes hæmorrhagic.

Microscopical examination of the tissues confirms these observations. The key to the pathological changes in the organs is found in the destructive action of the silica sol on the endothelial lining of the blood capillaries. This is shown frankly by the occurrence of petechial hæmorrhages, and is demonstrated in sections of organs, especially the liver, in which the vascular endothelium is found in different stages of degeneration, from simple swelling with vacuolation of cytoplasm to desquamation and often complete destruction. We do not wish to draw the conclusion that endothelium alone is poisoned by the sol, and that other pathological effects are merely a consequence of endothelial damage. Other cells are doubtless affected directly by the poison, but from the microscopical findings it is clear that the primary effect is on the vascular endothelium, which is to be expected from the mode of administration. Some of the changes observed are shown in Fig. 1. Large numbers of endothelial cells pass into the general circulation, and are seen in cross sections of blood-vessels. Fig. 2 represents a vein in the kidney, in which 9 of these large cells are seen. The picture is fairly representative of what may be seen in sections of any organ.

The question now arises: Are the lesions observed due to the action of bacteria? In the liver a few Kupfer cells are found containing large numbers of cocci, but the striking feature in respect of bacteria is the occasional occurrence of small masses of cocci—veritably colonies of organisms—in the capillary blood-vessels. They are most frequent in the kidney, liver and spleen, but may be found by persistent searching in most of the organs. The colonies are set in what appears to be altered blood; there is little or no tendency to wander through the capillary walls into or between adjacent cells; and there is no reaction in the tissues round about the masses. With iron hæmatoxylin they stain a more or less uniform black; the individual organisms are best demonstrated by Murray's (1919) Nile-blue picrofuchsin method of staining. Fig. 3 represents two glomeruli containing these colonies of bacteria; they must not be taken, however, as typical of the mass of glomeruli in a kidney, the vast majority exhibiting the necrosis of the tuft, or blood and serum in the capsular space, but containing no bacteria. In the case of two animals whose tissues were fixed immediately after death the bacterial masses have not been found.

The formation of these masses of organisms is undoubtedly a terminal phenomenon. The silica sol injected was sterile, and all the usual aseptic precautions were used in performing the operations. Further, the first animal of the series (No. 1 in the table above), after receiving thirteen injections, shows none of these colonies in liver, kidney or spleen.

It has already been stated that the liver and kidney show degenerative changes obvious to the naked eye. The special incidence upon these organs may be due to an attempt on their part to excrete the silica. The liver, in most cases, shows focal necrosis, sometimes to such an extent that more than half the liver parenchyma is dead; in two cases the amount of necrosis was



very small. The distribution of the necrotic areas is not characteristic. It is most frequently in the mid-zone of the lobules, though sometimes the lobule is completely destroyed. There are two kinds of necrosis present, and these may be found side by side. In one the liver-cells are represented by cell *débris*—cell “ghosts”—the columns being widely separated by dilated sinusoids which are packed with altered blood. The general appearance produced is that of a red infarct, but the mode of production of the lesion would appear to be that thrombosis has occurred in the capillaries, with subsequent death of liver-cells.

In the second type of necrosis the liver-cells are not completely destroyed; pyknotic nuclei and vacuolated protoplasm remain. The sinusoids are dilated but contain little or no blood; the endothelial lining is swollen and desquamating. In all cases the areas of necrosis (and in foci in the livers which do not show advanced necrosis) an extreme degree of fatty degeneration is observed. The liver-cells of the normal rabbit show, as a rule, small widely separated particles of fat which are generally situated near the cell boundary; the normal liver endothelium contains fatty particles quite as frequently as the liver-cell proper. Here and there endothelial cells may be found, of which almost the whole cytoplasm stains with sudan. In the degenerated livers of these experiments, however, the stainable fat in the dead and dying liver-cells and in many endothelial cells is enormously increased in quantity and occurs in large globules.

The most conspicuous damage to the kidney is shown by the glomeruli, which are universally affected. The smallest changes observed consist in an extraordinary dilatation of capillaries in the tuft with an exudation of serum into the capsular space; the epithelium lining the capsule is very prominent, often swollen and sometimes desquamated. In the more violent changes the tuft may be completely necrosed, and, as has already been said, colonies of bacteria may be shown in the midst of coagulated blood. Red blood-corpuscles are found in the capsular space and appear in the form of casts in the tubules. The renal epithelium, although showing degeneration here and there, is not so badly affected as would be expected from the violence of the changes in the glomeruli. There is a mild degree of fatty degeneration, which, however, is not so conspicuous in the highly specialised cells of the convoluted tubules as in the cells of the collecting tubules. The ducts of Bellini often show extensive fatty degeneration. Fat is also found in the glomerular tufts. Enormous numbers of casts are found in the tubules, many of which are widely dilated.

The histological changes in the spleen are somewhat variable. In most of the spleens a necrotic effect predominates; in a few an extreme congestion of the organ is most noticeable. In the former type there is an evident disappearance of cells, those remaining being widely separated by a swollen reticulum in which particles of chromatin are found; the lymphoid masses are scanty and often show areas of focal necrosis. In the blood channels large numbers of mononuclear cells with abundant cytoplasm which often contains inclusions are found. In the second type the blood sinuses are distended with blood which contains large numbers of endothelial cells. There is not such evident destruction of cells as in the form already described.

The pathological changes found in the other organs are not sufficiently

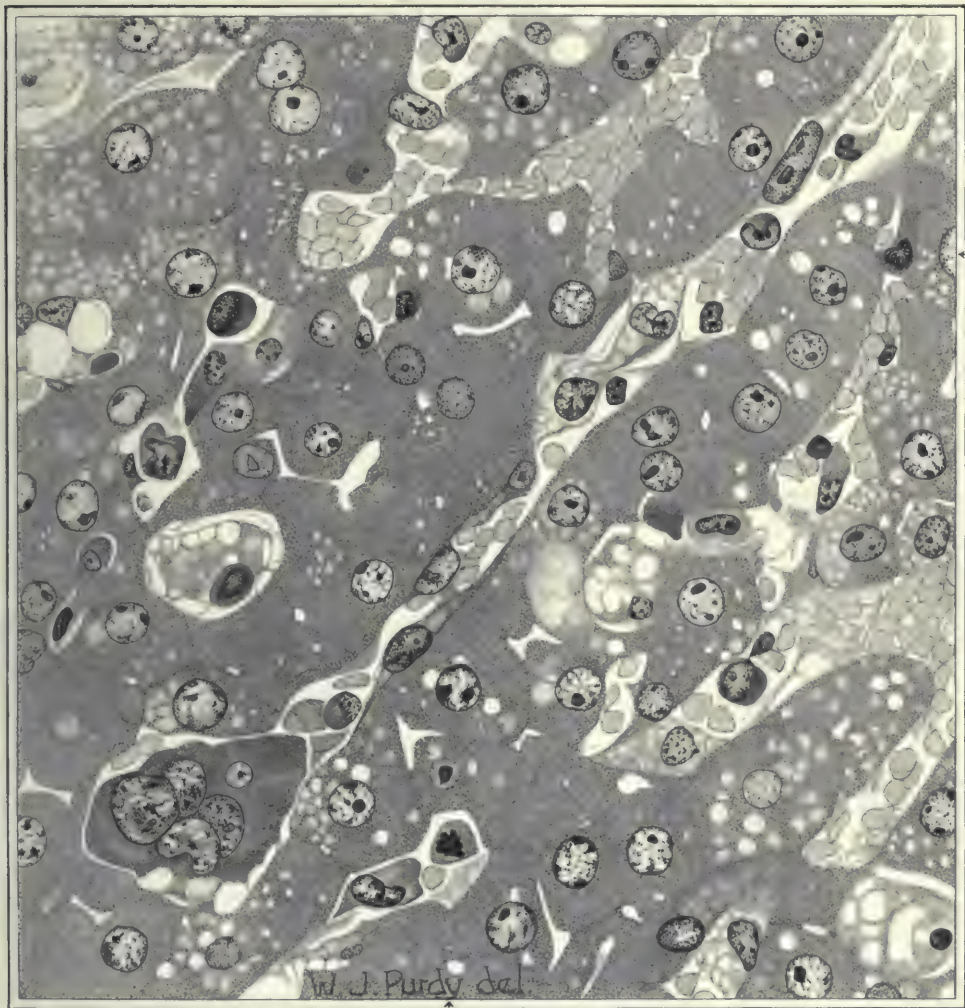


FIG. 1. Scale 830:1.

*Stained iron-haematoxylin and van Gieson.*

Liver of Rabbit 76/A which received intravenously for 4 days daily doses of 24 mgrm. of silica. The endothelial cells are increased in number and are considerably changed. They show various degrees of enlargement, vacuolation, and desquamation. Some of those which are free have deeply-staining cytoplasm. Giant-cell formation is seen. Mitotic figures are present in 2 endothelial cells—one opposite each marginal arrow. The liver-cells in the area figured do not show the more extreme changes seen in other parts of the section.





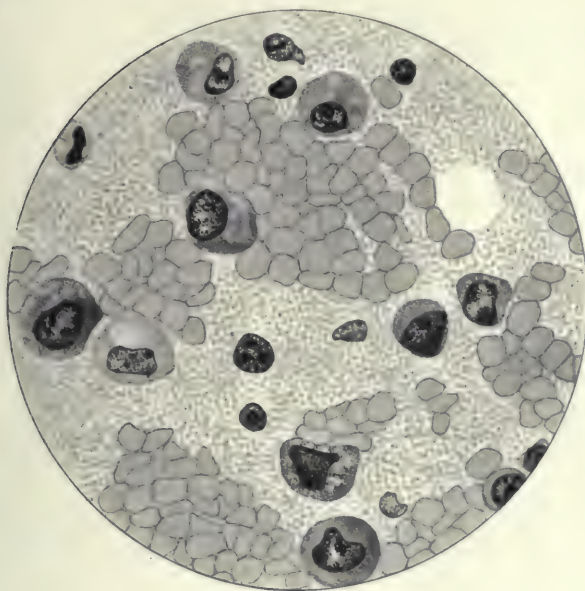


Fig. 2. Scale 1100:1.  
*Stained iron-haematoxylin and van Gieson.*

Blood in a large vessel in the kidney of Rabbit 71/A which received intravenously 72 mgrm. of silica on each of 3 successive days. Nine large, free endothelial cells are seen in the section. One is faint and considerably vacuolated, while the others have deeply-staining cytoplasm and a tendency to vacuolation. Similar cells are present in the blood in sections of the other organs.

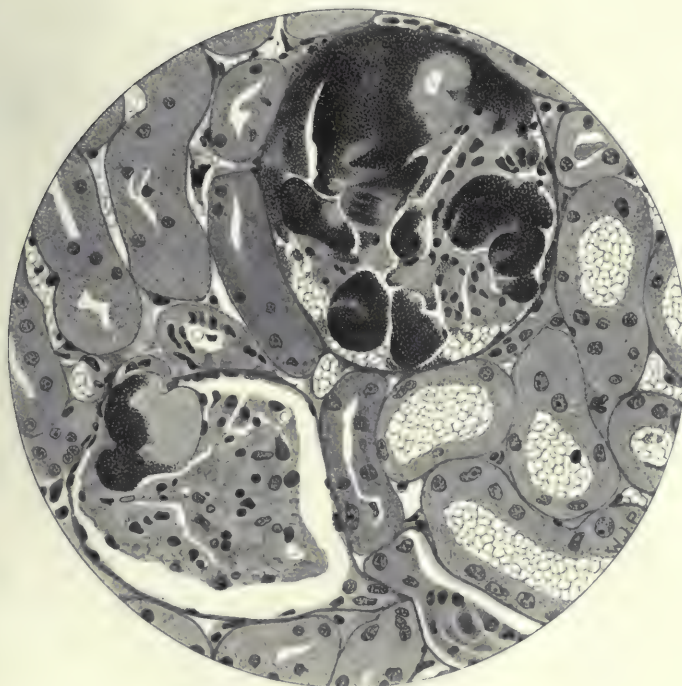


FIG. 3. Scale 360:1.  
*Stained iron-haematoxylin and van Gieson.*

Kidney from the same animal as is the preceding figure. The glomerular tufts show dilated capillaries filled with altered blood in which colonies of cocci have grown. The tufts also show a good deal of necrosis and loss of vascularity. There is blood in the capsular space of the upper glomerulus and also in several tubules. The tubular epithelium shows some cytoplasmic and nuclear change.





regular to deserve detailed description. It has already been stated that hæmorrhages may be found in any situation, and reference has been made to the condition of the ureters in two animals. The mucous membrane of the alimentary tract exhibits petechial hæmorrhages almost constantly. In one animal the cortex of the suprarenal bodies is necrosed and in others small hæmorrhages are found. But, generally speaking, apart from the occurrence of petechial hæmorrhages, the other organs of the body are not constantly involved in the necrotic process observed in liver, spleen and kidney.

## SUMMARY.

Silica sol administered parenterally is a poisonous substance. Injected intravenously in very large doses it causes immediate death, which is due to clotting of the blood. Injected in sublethal doses it accelerates the rate of clotting of the blood when shed. Added to blood *in vitro* it has no influence on the rate of clotting. Silica sol injected intravenously in rabbits in daily doses of 30 to 72 mgrm. causes death in two to four days, and *post-mortem* petechial hæmorrhages and profound degeneration of liver and kidney are found. The conclusion is drawn that the primary action is on the vascular endothelium.

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# THE POISONOUS PROPERTIES OF COLLOIDAL SILICA.

## II: THE EFFECTS OF REPEATED INTRAVENOUS INJECTIONS ON RABBITS; FIBROSIS OF THE LIVER.

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In the previous paper (1922) it has been shown that silica sol, administered parenterally, is a poisonous substance, and the conclusion has been drawn that it acts on the vascular endothelium. When rabbits are injected intravenously with daily doses of 30 to 60 mgrm. they lose appetite, become lethargic, and die in 3 or 4 days with extensive degeneration of the liver and kidneys.

In the present paper we shall describe the results obtained by injecting rabbits at daily or weekly intervals with doses varying from  $\frac{1}{4}$  to 30 mgrm. The large doses have been given at weekly intervals. For the sake of conciseness and convenience the doses and intervals are given in the following table of the experiments which are described in this paper.

Dose.	Interval.	Number of animals.
$\frac{1}{4}$ mgrm.	Daily	2
5 "	"	8
10 "	"	3
5 "	Weekly	1
30 "	"	3

In addition to this list of 17 animals, others have been used in variations of such experiments as the above; the results obtained with these are merely confirmatory of what is to be described, and are omitted from this paper merely for economy. Further, the results have been controlled by the examination of an equal number of normal animals.

The general health and well-being of all the rabbits treated with the doses and at the intervals given in the table above have remained perfectly satisfactory. Whilst a daily dose of 30 mgrm. kills a rabbit in a few days, a daily dose of 10 mgrm. has no apparent effect even over a period of months. We have under observation at present an animal which has been injected daily for over 7 months with 10 mgrm. of  $\text{SiO}_2$  in the sol form, and which has been throughout, and still is, in perfect health; it has increased in weight, its

appetite is good, and it betrays no weakness or loss of vitality. Examination of the urine, however, reveals the presence of albumin—which has come on since the experiment began—casts, and occasionally red blood-cells and crystals of leucin and tyrosin. But, speaking generally, the rabbit would be accepted as normal for experimental purposes. The state of the urine is the only indication of the slow anatomical changes which are occurring under the influence of the daily injections of silica sol.

There is thus a fairly sharp line in dosage above which the lesions caused by silica are so great that daily repetition of the dose leads to such profound degeneration of vital organs as to be inconsistent with life, and below which the dose may be repeated daily without any obvious disturbance of health. In this latter class of experiment the main pathological effect is the production of new connective tissue, especially in the liver, but also in the kidney and spleen, together with other changes which will be dealt with later.

The three rabbits which have been injected weekly with 30 mgrm. of silica—3 c.c. of 1 per cent. sol made isotonic immediately before use—demonstrate a point of some importance. The fact that these rabbits survive—and not only survive, but remain in good health—after such treatment shows, when taken in conjunction with the results of experiments in which 30 mgrm. are injected daily, that the lesions produced by colloidal silica are rapidly recovered from. In one week the effects of a 30-mgrm. dose have so far passed off that the animal, in respect of the succeeding dose, behaves like a normal. The intense effects of large doses are not observed, therefore, unless the intervals of time between the injections are short.

Before passing on to the closer consideration of the individual animals of the several experiments, reference must be made to the degree of variation found in the histology of the normal liver and kidney in rabbits. Since the principal alteration in structure in the animals in these experiments consists in the laying down of new connective tissue in the liver, with little alteration in the parenchyma, the variations among cells have not been closely studied. We confine ourselves to the consideration of the simplest elements of histology, leaving to the future the more refined analysis of the changes which take place.

Orphüls (1908), in an interesting paper, which contains an able plea for the experimental study of chronic non-bacterial diseases, makes the remark that “rabbits are practically useless in work on cirrhosis, because coccidiosis is so common among them, and even when healed leaves more or less marked traces in the liver.” The experience on which this opinion is based is given in greater detail in a subsequent paper (1910). Orphüls examined the livers of 50 rabbits, some young and some old, some bought from the markets and some raised in the laboratory; many of the animals had been used for experiments in which no injury to the liver had been caused; a few had died of coccidiosis. In six only was the liver perfectly normal. The rest showed various stages of coccideal infection with more or less cirrhosis, and in some cirrhotic processes were observed though coccidia could not be demonstrated. The percentage of animals suffering from coccidiosis is not given, though, judging by the emphasis laid upon this infection, it was high. We are unable, in spite of the figures, to agree with Orphüls’ extreme opinion, and for several reasons. In the first place the frequency with which coccidiosis occurs depends



upon the amount of care taken in the housing and feeding of rabbits. The disease can be reduced to negligible proportions by proper attention to hygienic conditions and prompt elimination of weakly animals. In the series of 14 rabbits described in this paper 2 were infected with coccidia, and these only to a small extent; the remaining 12 were examined carefully and the livers were found to be free. In other series of animals, *e.g.* those described in our previous paper, coccidiosis was absent. In the second place, where the liver is infected, the lesions produced are circumscribed, and, unless the organ is simply riddled with coccidia, no difficulty should be experienced in fixing their limit. And finally, our experience in these experiments has been that rabbits which are injected with small doses of silica sol for five weeks or longer always show fibrosis of the liver, whereas, with one exception, control animals which are fed and otherwise treated in exactly the same way do not. The difference has been quite sharp. Moreover the character and extent of the fibrosis are consistently correlated with the size of dose of silica and with the length of time during which the injections are continued, and are always accompanied by alterations in the spleen and kidney. Although rabbits are not ideal animals for the study of cirrhosis of the liver, they cannot be rejected, especially in experiments involving repeated intravenous injections.

The changes found in the kidneys in this part of our work are less striking than those found in the liver. The primary lesions are found in the glomeruli, which may show simply dilatation of capillaries and diapedesis of red cells into the capsular space, or more advanced changes, such as hæmorrhages or necrosis of the tuft. In rabbits which have been under experiment for many months, thickening of the glomerular capsules and increase in the intertubular connective tissue are observed. Parenchymatous changes are less conspicuous. The changes we shall describe are certainly not mere normal variations—they are a regular consequence of the injections of silica.

Orphüls, in the series of 50 rabbits already referred to, found that 28 had normal kidneys, 9 showed slight parenchymatous changes, 3 showed small areas of cellular infiltration, and in 10 there was a definite increase in the interstitial tissue. Lesions in the glomeruli and blood-vessels were never seen. Longcope (1913) examined 24 normal animals: in 16 the kidneys were "absolutely normal"; in 4 "well-marked fibrosis scars" were found in the cortex, and in 4 there were patches of small round cells between the tubules of the cortex. "In no instance were glomerular lesions encountered." Our own observations on the kidneys of normal rabbits agree substantially with the above, but it must be added (1) that casts are sometimes found, (2) that in one instance we have seen small masses of red blood-cells in collecting tubules, and (3) that in the kidneys of two normal rabbits occasional glomeruli were fibrosed.

We now pass on to a consideration of the individual animals of the experiments.

#### A. *Experiments in which 10-mgrm. Doses were Injected.*

Three animals were used; two have been killed and one is still alive and under observation.

*Rabbit 41/1.*—This animal was injected daily for 16 weeks. The total weight of silica given was 0.94 gm. The weight of the animal at the beginning of the experiment was 1600 gm., and at the end 3150 gm. The gain in weight of 1550 gm. in 16 weeks is good evidence that the animal's general health was unaffected. The urine was examined at the beginning of the experiment, when there were a few casts and epithelial cells in a centrifuge deposit, but no albumin was present. After 5 weeks albumin appeared together with red blood-cells and an increased number of casts.

The rabbit was killed with coal gas and a *post-mortem* examination made at once. The liver was of average size; the surface was rough and granular, with bands of fibrous thickening stretching across individual lobes; it was firm and was tough and resistant to the knife. On the cut surface the lobules stood out prominently. There were no coccideal lesions in the organ. The spleen was greatly enlarged and patches of perisplenitis were conspicuous. The kidneys, pancreas, adrenals, heart and lungs, thymus and thyroid showed no naked-eye changes. Several tape-worm cysts were noted in the mesentery.

Microscopical examination of the tissues: The liver shows a considerable fibrosis. The thickest and best-formed bands of fibrous tissue are found in and immediately under the capsule of the organ and in tracts connecting the portal spaces. Finer tracts of fibrous tissue are found radiating from the intralobular veins and running between the columns of liver-cells. In places this intralobular connective tissue is thick enough to be observed easily under a low magnification (*e. g.* 60 $\times$ ); generally, however, the strands are fine and delicate and are only distinctly visible under a  $\frac{1}{4}$  objective. The type of fibrosis may be described as mixed, fine and coarse. The liver-cells are healthy. Here and there the newly-formed connective tissue is found surrounding individual cells or groups of cells, but there is no destruction of the cells.

The kidney shows lesions of the glomeruli; the tubular epithelium is unaffected. The glomeruli show thickening of the capsule, which has, in a few instances, advanced far enough to involve the tuft, the whole structure being fibrosed. Multiplication of the epithelial cells lining the capsule is often found. The capsular space sometimes contains albumin and blood; the tufts show an increased cellularity. There is an increase in the intertubular connective tissue, especially in the cortex; bands of fibroblasts are found dipping down from the capsule and running to the medulla (Fig. 8). Casts in tubules are fairly common.

The great enlargement of the spleen (Fig. 9) is due to congestion and an increase in the amount of connective tissue. The capsule is thickened, the fibrous trabeculae of the organ are very prominent, and there are numerous bands of young fibroblasts stretching from the capsule to trabeculae and forming a network through the organ.

The heart, lungs, pancreas and thyroid, thymus and adrenals are normal.

*Rabbit 41/3.*—This animal was small and not so active as the other two of the experiment. It was killed after 18 weeks of daily doses of silica; the total weight of silica given was 1.02 gm. During the first 8 weeks of the experiment the animal, though not definitely ill, was not active; its weight remained stationary, and its appetite was not good. After the eighth



week it began to improve and put on weight rapidly. At the beginning of the experiment it weighed 1050 grm., when killed 18 weeks later 2700 grm. Thus as compared with rabbit 41/1 the dose of silica given was, for 8 weeks, weight for weight, roughly double. As the rabbit increased in weight the dosage approximated to that of the other animals of the experiment. The urine was examined from time to time, and was found to contain albumin, casts, red blood-cells and epithelium, occasionally bile and crystals of tyrosin and leucin.

*Post-mortem examination*: The liver was of average size; the surface was very rough and granular, and the capsule obviously thickened; it was firm and cut with great resistance. There were a dozen circumscribed white spots on the surface or in the substance of the organ which were due to coccidia. The spleen was greatly enlarged and was firm. The kidney, pancreas and other organs were not conspicuously altered.

*Microscopical examination*: The liver shows a multilobular cirrhosis with a tendency for the fibrous tissue to advance between the columns of liver-cells. The capsule of the organ is very thick, and immediately under it there are liver-cells crushed and atrophied by bands of fibrous tissue which pass from the capsule towards portal tracts and unite to form a dense network. The bands of fibrous tissue surrounding the liver lobules are in places half the width of a single lobule. Thus the fibrous framework of the liver is enormously increased. At the edges of the fibrous bands the liver-cells become surrounded with connective tissue and appear to be strangled. There is a multiplication of bile-ducts in the fibrous tissue. These changes are shown in Figs. 3 and 4, in the first of which the general appearance of the liver in a very thin section is shown, and in the second the proliferation of bile-ducts.

The splenic enlargement is due to congestion and to a multiplication of reticulum cells and a general fibrosis. The kidney shows similar changes to those described in animal 41/1. Other organs are not affected.

Rabbit 41/2 is still under experiment.

#### B. *Experiments in which 5 mgrm. were Injected Daily.*

Eight animals have been employed. Of these four were killed within 16 days—one on the tenth, one on the twelfth, one on the fifteenth, and one on the sixteenth days. These may be taken as one group. They were killed for the purpose of studying the earliest changes which occur—a study which is not completed. They exhibit no increase in fibrous tissue either in the liver or kidneys, and in this respect may be added to the list of control animals. Of the remaining four rabbits, one was killed 6 weeks after the beginning of the experiment, two 8 weeks after, and one 27 weeks after. These will now be taken in the order given.

*Rabbit 40/3*.—The animal remained perfectly well during the 6 weeks. It received altogether 140 mgrm. of  $\text{SiO}_2$ . Its weight increased from 1550 to 1600 grm.

*Post-mortem examination*: The surface of the liver was granular and rough. The organ was firm and more resistant than normal on cutting; the cut surface was rather paler than usual. The spleen was enlarged. The kidney,

pancreas and other organs showed no abnormalities. There were no tape-worm cysts and no coccidiosis.

The microscopical examination of the liver revealed a fine fibrosis. Delicate strands of connective tissues are seen penetrating between the columns of liver-cells; in some places, and especially immediately beneath the capsule, individual liver-cells are seen surrounded by fibrous strands. The capsule is thickened, and from it rather denser bands of fibrous tissue dip into the substance of the organ. The kidney shows in section extensive damage of the glomeruli. The capillaries of the tuft of most of the glomeruli show dilatation; a small proportion are necrosed. There is serum and often blood in the capsular space, and blood-casts are common in the tubules. There is no increase in the interstitial connective tissue.

The enlargement of the spleen is due to an extreme congestion and possibly partly to a multiplication of reticulum cells.

*Rabbits 18/1 and 18/2.*—These animals, killed after 8 weeks, each received 210 mgrm. of  $\text{SiO}_2$ . They increased in weight during the experiment, one from 1750 to 1900 gm., and the other from 1500 to 1700 gm. *Post-mortem* neither animal showed either tape-worm cysts or coccidiosis. The urine taken from the bladder after death contained no albumin and no blood, but numerous crystals of tyrosin were present.

The liver of each animal was firm and had a finely granular rough surface. The spleens were enlarged, the other organs were normal to the naked eye.

Microscopical examination of the tissues shows a fine fibrosis of the liver, rather more advanced than, but similar in character to, that found in the last animal (40/3) described.

The enlargement of the spleen is due mostly to extreme congestion. The kidneys show congestion of most glomeruli with serum in the capsular space and in a few complete necrosis of the tuft.

The last animal of this series, 40/4, was kept under experiment for 27 weeks. It received altogether 0.790 gm. silica. During the experiment the animal's weight rose from 1300 to 3100 gm. The urine was examined from time to time, and a trace of albumin was found together with red blood-corpuses, epithelial cells and casts.

*Post-mortem* examination: The surface of the liver was rough; the liver was firm and resistant when cut. There was no coccidiosis. The spleen was enlarged; the kidneys slightly pitted and rough on the surface. There was a small amount of fluid in the pleural cavity and a relatively larger amount in the pericardium. The surface of the left ventricle was distinctly roughened. The lungs, thymus, thyroid, pancreas and adrenals appeared normal.

Microscopic examination: The capsule of the liver is greatly thickened; immediately beneath, thick bands of fibrous tissue form a network in which islets of liver-cells are caught. The portal spaces are joined together by new connective tissue which spreads in between the columns of liver-cells, forming, in some places, solid tracts, in others more delicate cellular strands. The extent of the fibrosis is not, however, so extensive as in rabbit 41/3 or 41/1, already described.

The enlargement of the spleen is due to congestion, multiplication of spleen cells and fibrosis; the capsule is extremely thick. The kidney is



relatively more damaged than the liver comparing this rabbit with those already described. The capsule is thick, and strands of fibrous tissue, which correspond in position with the surface depressions already noted, pass into the organ to the medulla; the fibrous tissue passes in between tubules and surrounds the glomeruli encountered in its track. There is thus a pronounced increase in the interstitial tissue. The capsules of almost all the glomeruli are thickened; in a fair proportion the process has extended to the tufts, the whole Malpighian body being sclerosed. The tubular epithelium is not affected. The pathological picture presented is that of an early interstitial nephritis.

The heart-muscle shows an increase in fibrous tissue, an interstitial myocarditis.

The other organs are not noticeably affected.

*c. Experiments in which  $\frac{1}{4}$  mgrm. was Injected Daily.*

Two rabbits were employed; one was killed after 14 weeks, the other after 28 weeks. The first received 57 doses in all, the total weight of silica injected being 15 mgrm. Its weight remained practically constant (at 2600 grm.) during the experiment, the animal being full grown. *Post-mortem* there were no signs of disease in any organ. Microscopical examination of the liver shows the existence of delicate strands of young connective tissue stretching between portal tracts. The amount of connective tissue is very small, and appears of significance only when looked at in relation to the results of other experiments. The kidney, spleen and other organs are unaffected.

The second rabbit, treated for 28 weeks, received altogether 42 mgrm. silica. During the whole period of the experiment the animal remained in good health; its weight increased from 2050 to 3350 grm. Traces of albumin, casts and red blood-corpuscles were detected in the urine 5 months after the commencement of the injections, and were observed again on three out of the four times that the urine was tested.

*Post-mortem* examination: The liver showed a patchy perihepatitis, the surface was granular; the organ was firm and creaked when cut. There were no coccideal lesions. The spleen was enlarged. The thyroids were enlarged. The other organs showed no definite naked-eye lesions.

Microscopical examination of the liver demonstrated a great thickening of the capsule, and the existence of firm, well-formed fibrous bands distributed around the lobules and giving to a section an appearance similar to that of a pig's liver (Figs. 5 and 6). There is practically no tendency for the connective tissue to pass in between the liver-cells towards the intra-lobular vein. The liver-cells, judging by their appearance, are perfectly healthy.

The spleen shows an increased thickness of the capsule, the presence of abnormally thick fibrous bands running through the organ, and considerable congestion.

The capsule of the kidney is definitely thickened with well-formed fibrous tissue. Some of the glomeruli show thickening of the capsule; there is an increase of interstitial tissue. The tubular epithelium is not damaged.

*D. An Experiment in which 5-mgrm. Doses were Injected Weekly.*

The rabbit used in this experiment had been in the laboratory under observation from the time it was 8 weeks old. When the experiment was begun it was full grown and weighed 2650 grm.; its weight at death was 2900 grm. During the whole period of the experiment, which lasted 29 weeks, the animal was in good health. The total weight of silica injected was 140 mgrm. The urine was tested from time to time, and for the first 5 months albumin, blood-cells and granular casts were absent. They began to appear after the twentieth week, and were afterwards constantly present. Thus the urine was examined four times during 14 days, and on each occasion albumin, occasional red cells and granular casts were present.

At the *post-mortem* examination the rabbit was found in good condition, fat and free of coccidiosis and tape-worm cysts. The liver had a somewhat roughened surface, and was firm to the touch. The kidneys were normal in size, but the cut surface was rather granular. Pancreas, spleen, lungs and heart, adrenals and thyroid glands were normal.

On microscopical examination it was found that the excess of fibrous tissue in the liver was almost confined to the capsule. In the substance of the organ there is a small overgrowth of connective tissue between the portal spaces, and delicate collagenous fibrils are shown within the lobules, generally radiating from the central vein. On the whole, however, there is a negligible increase compared with the animals of the other experiments.

The kidneys show a thickened capsule, an increase in interstitial tissue, and changes in some of the glomeruli, thickening of capsule and serum in the capsular space (Fig. 7).

*E. Experiments in which Weekly Doses of 30 mgrm. were given.*

Of the three rabbits submitted to experiment one was killed after 16 weeks; the other two are still under observation.

*Rabbit 64/1.*—The animal increased in weight from 1850 to 3000 grm. during the 16 weeks of experiment.

The urine was not examined.

*Post-mortem* examination: The liver was granular on the surface and firm on section. There were several pin-head white spots of coccidiosis on the surface and in the substance of the organ. The spleen was abnormally short, but was greatly increased in size. The remaining organs were normal to the naked eye.

Microscopical examination: There is a moderate degree of fibrosis of the liver; the capsule is thick, and the portal tracts are connected by fibrous tissue, which has no tendency to pass between the liver-cells towards the centres of the lobules.

The enlargement of the spleen is caused by congestion and an increase in the fixed cells of the organ.

The kidney shows a moderate amount of glomerular damage, consisting of exudation in the capsular space and frequently thickening of the capsule. There are areas which show an increase in interstitial tissue. Casts are found abundantly in the tubules, but there is no degeneration of tubular epithelium.



*Note on the Pathological Anatomy of the Lesions.*

The description of the microscopical findings in the different animals has now been given briefly. For the finer details the reader is referred to the accompanying drawings, which illustrate accurately the distribution of the new connective tissue, and at the same time show the cellular changes which have been observed. Fig. 1 is the key to a proper understanding of the course of events which ends in fibrosis. In this figure (and to a less extent in Fig. 2) it may be observed that the endothelial cells lining the liver sinusoids are unusually numerous; many are large and vacuolated, and some are completely degenerated; the new fibrous tissue forms a thick wall to the capillaries. The figure should be compared with Fig. 1 of Part I of this series of papers, in which an acute and more extensive cellular degeneration is exhibited, but which shows no fibrosis. Fig. 1 of this present paper illustrates the slower, less violent change, the new connective tissue being formed as a defensive or repair process in response to the endothelial damage.

## SUMMARY.

The intravenous injection of 5 mgrm. or more of silica sol daily in rabbits is followed by fibrosis of the liver, enlargement of the spleen, and changes in the kidney resembling interstitial nephritis.

The experiment in which  $\frac{1}{4}$  mgrm. was injected daily is open to the criticism that too few rabbits were employed. We give the results, therefore, without further comment.

The weekly injection of 5 mgrm. of silica sol has but little effect on the liver and kidney, but in both organs the changes found, though small, are quite definite. One animal only has been used in this experiment, and the conclusions drawn are, therefore, provisional. It would appear from this single result that, in order to cause an abundant formation of connective tissue with this dose, the injection of silica must be repeated daily. If seven days are allowed to elapse between the administration of the doses, the lesions produced by each dose heal practically completely before the next dose is given. When the weekly dose is 30 mgrm. a very definite fibrosis of liver and degeneration of kidney are produced in a few months. (The single result reported in this paper has been confirmed in other experiments, which will be published later.) From this it may be concluded that the damage to cells caused by 30 mgrm. is too extensive to be healed completely in one week, and that a chronic pathological condition is induced, fibrous tissue being formed in the constant attempts at repair.

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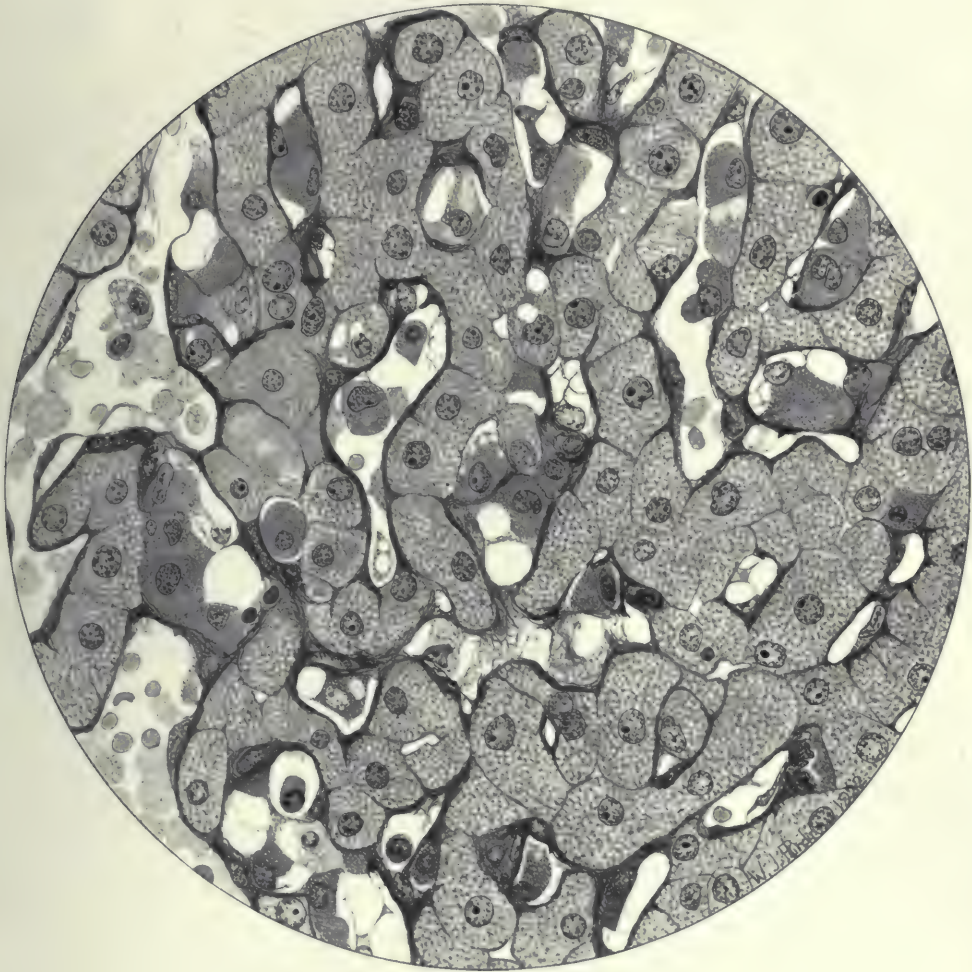


FIG. 1. Scale 700 : 1.

*Stained iron-haematoxylin and van Gieson.*

Liver of Rabbit 40/3, which received intravenously for 6 weeks daily doses of 5 mgrm. of silica. Notice the endothelial cells in the dilated sinusoids. They are increased in numbers and most, or all, in this part of the section are altered and show various degrees of enlargement, vacuolation, and desquamation. Note, too, how the new connective tissue forms a thick lining to the sinusoids and tends to spread thence in between the individual cells of the columns. Compare with the sinusoid walls in Fig. 6 or, preferably, with those in Fig. 1 of Part 1, which show no perceptible connective tissue in this situation, and which are, in this respect, normal.





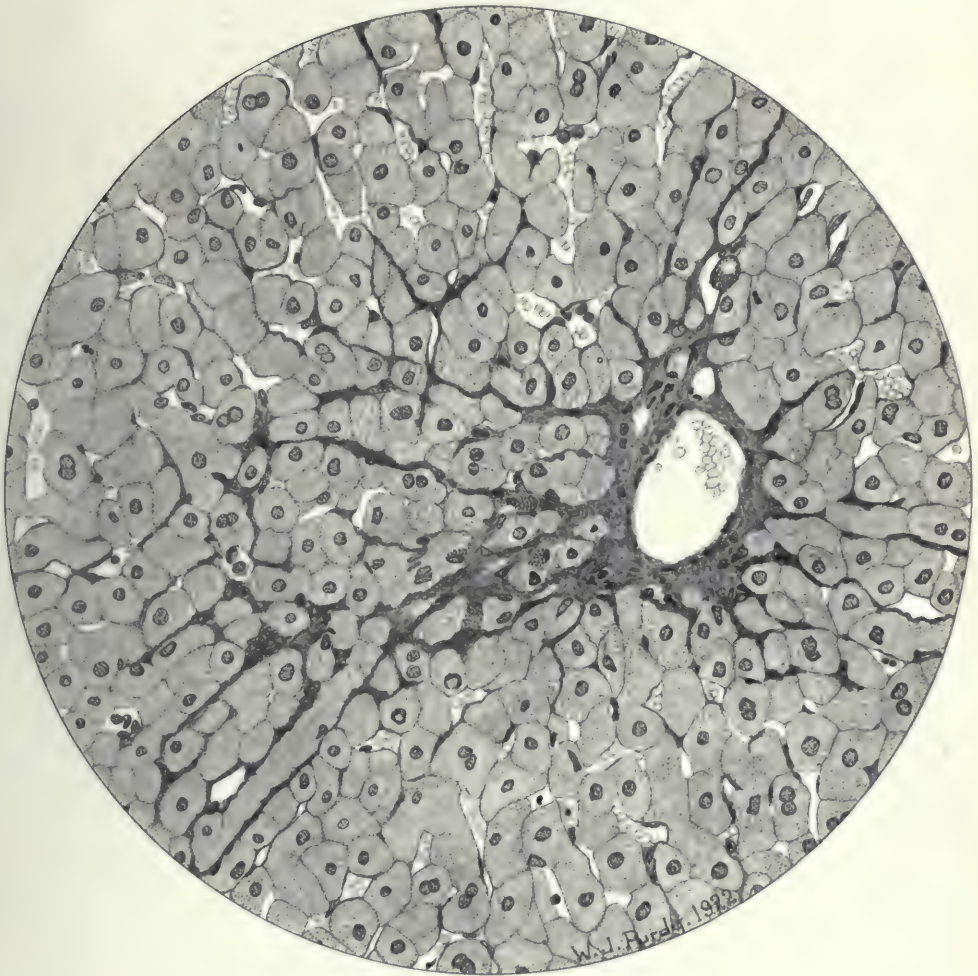


FIG. 2. Scale 320 : 1.

*Stained iron-haematoxylin and van Gieson.*

Liver of Rabbit 41/1, which received intravenously for 16 weeks daily doses of 10 mgrm. of silica. New-formed fibrous tissue is seen around a central vein and stretching between the columns, and even between the individual cells. Where the relationship to vessels can still be made out, it will be seen that this new connective tissue appears in the sinusoid walls, though the process is not so regular as that found in Fig. 1, the thickening being often confined to one part only of a wall.





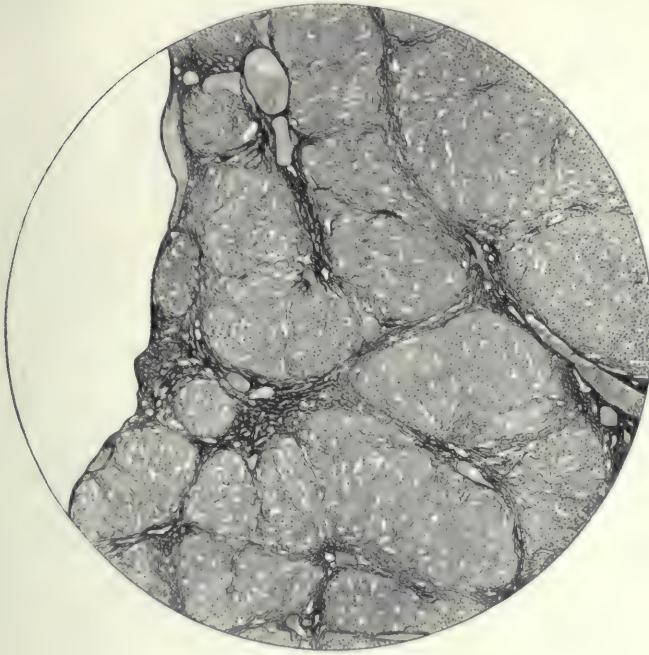


FIG. 3. Scale 24 : 1.  
*Stained iron-haematoxylin and  
van Gieson.*

Liver of Rabbit 41/3, which received intravenously for 4 months daily doses of 10 mgrm. of silica. This drawing is from a very thin section and shows distinct fibrosis, mainly interlobular in distribution.

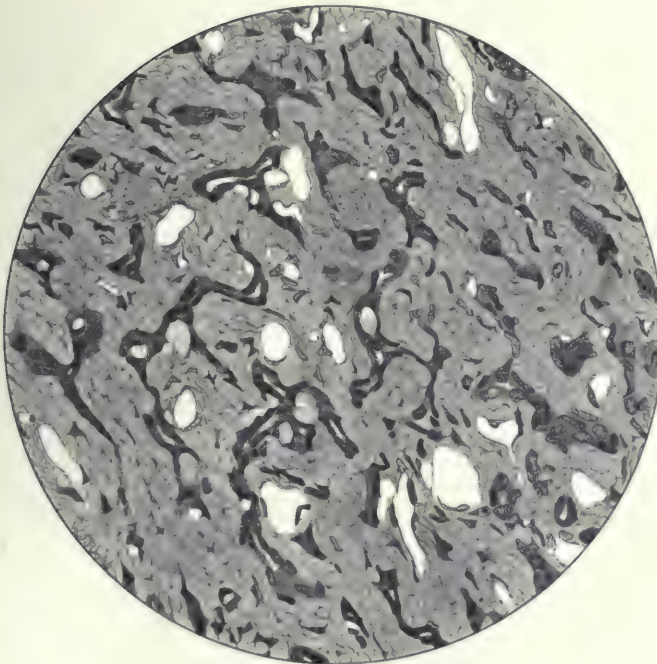


FIG. 4. Scale 440 : 1.  
*Stained iron-haematoxylin and  
van Gieson.*

A portion of a band of new-formed fibrous tissue in the liver of Rabbit 41/3. Numerous new-formed bile-ducts are seen. Fixation in Carnoy's fluid has caused a swelling of the collagenous fibres and they are not seen individually. Many of the vessels are lymphatics; others contain blood in the original section, but for the sake of clarity the corpuscles have been omitted in the drawing.





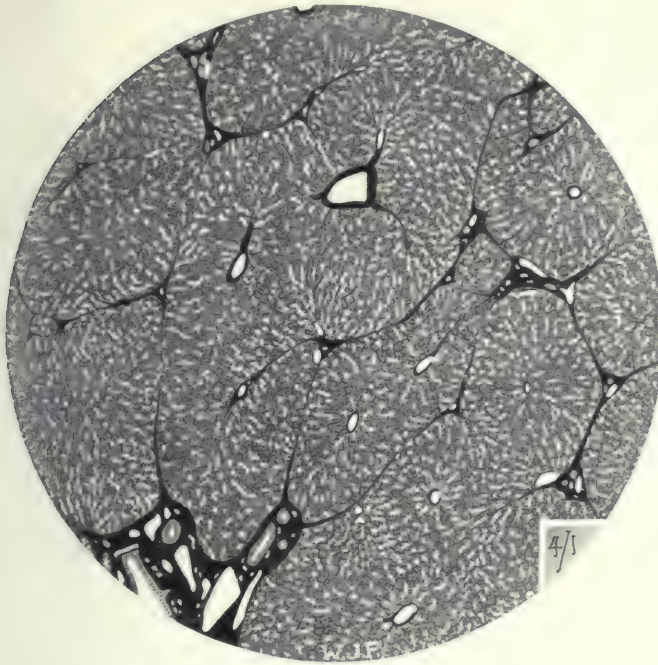


FIG. 5. Scale 22:1.  
*Stained iron-haematoxylin and van Gieson.*

Liver of Rabbit 4/1, which received intravenously for 7 months daily doses of 0.25 mgrm. of silica. There is a definite increase of connective tissue which appears in dense, well-formed bands, connecting up the portal tracts and surrounding the central veins. The fibrous tissue does not pass in between the cells and columns of the lobules, though in other parts of the section there is some tendency to do so.

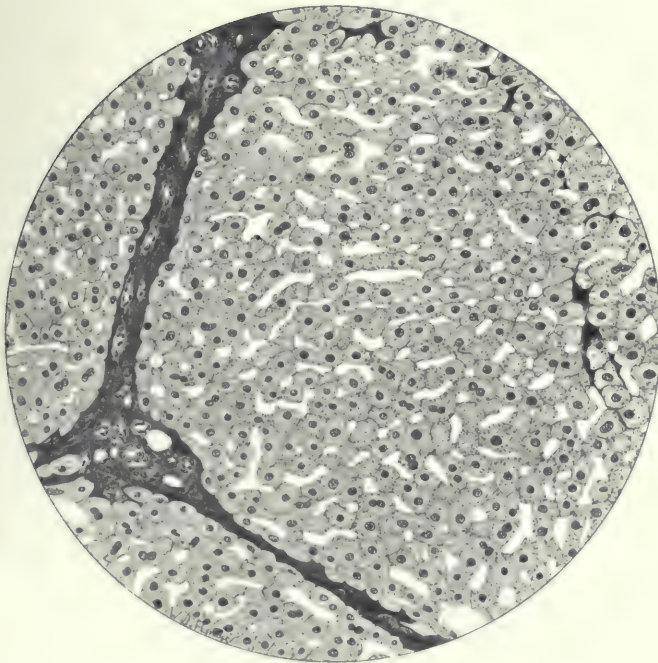


FIG. 6. Scale 170:1.  
*Stained iron-haematoxylin and van Gieson.*

The section is from the same animal as is the preceding figure. An interlobular band of connective tissue is seen in the liver. This is fairly representative of the fibrous tissue shown on a smaller scale in Fig. 5. It will be noticed how little is the tendency to spread between the cells and columns. Towards the right is a little intercellular connective tissue. The tissue was fixed in Carnoy's fluid, and so the fibrous tissue does not show individual fibres.





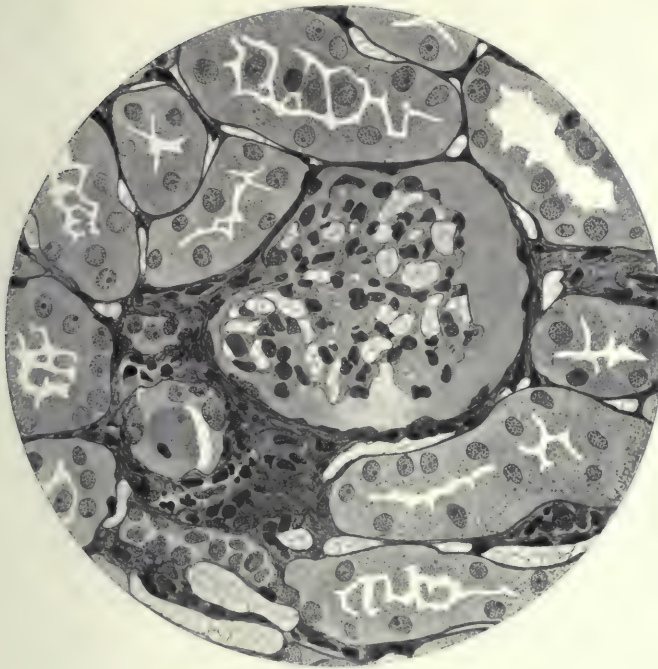


FIG. 7. Scale 470:1.  
*Stained iron-haematoxylin and van Gieson.*

Kidney of Rabbit 3/7, which received intravenously for 7 months weekly doses of 5 mgrm. of silica. The dilated capsular space is filled with albuminous material. Bowman's capsule is a little thickened. New-formed connective tissue is present beside the glomerulus. One tubule contains a cast of albuminous material.

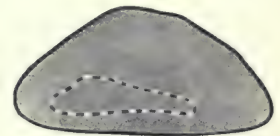


FIG. 9. Scale 2:1.

From the same animal as is Fig. 8. A transverse section of the spleen is represented, and on it the dotted line shows to the same scale the largest transverse section from amongst 6 consecutive normals. The other splenic dimensions showed corresponding enlargement.

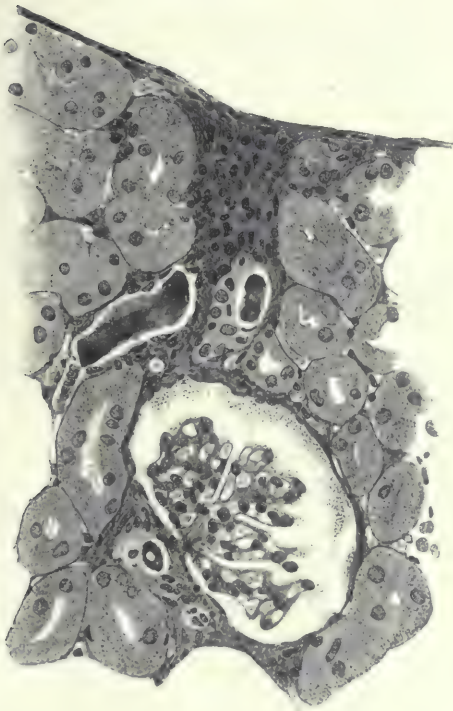


FIG. 8. Scale 300:1.

*Stained iron-haematoxylin and van Gieson.*

Kidney of Rabbit 41/1, which received intravenously for 4 months daily doses of 10 mgrm. of silica. A band of new-formed connective tissue is seen coming in from the capsule towards, and past, a glomerulus. In this band are several disappearing tubules, some of which contain casts. The dilated capsular space contains albuminous material.





## THE ASSOCIATION OF THE VIRUS OF TYPHUS FEVER WITH THE VARIOUS BLOOD ELEMENTS.

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As the virus of typhus fever has not as yet been definitely cultivated, one is obliged to make use of virus-containing material (*e. g.* blood and brain) from infected animals in all experimental investigations on this disease.

This is a great drawback, as much unnecessary protein (tissue cells, etc.) is added to the volume of the inoculum.

The desirability of having the virus, especially for immunisation purposes, in as pure and as concentrated a form as possible is obvious, and it was with this object that the following experimental work was carried out.

### ASSOCIATION OF THE VIRUS WITH THE LEUCOCYTES.

In 1912 Charles Nicolle brought forward experimental evidence to show that the leucocytes form the principal, if not the only infective or virus-carrying portion of the blood in typhus fever, the virus being apparently contained within these cells.

The observations supporting this assertion were as follows :

In a severe case of typhus fever, blood was taken on the seventh day of disease and distributed in sterile centrifuge tubes containing 2 per cent. citrate solution (18 c.c. blood to 2 c.c. citrate solution). Some of this blood was centrifuged during twelve minutes (speed not indicated) and the supernatant citrated plasma pipetted off and kept. The leucocytic layer of the deposit was then carefully removed and emulsified in saline. Similarly a few c.c. of red cells were collected from the deepest layer of the deposit and suspended in saline. These suspensions were again centrifuged during ten minutes and the supernatant fluid removed. Monkeys (*M. sinicus*) were then inoculated with—

- (1) 5.0 c.c. of the citrated plasma.
- (2) About 1 c.mm. of the leucocyte layer.

This material on microscopical examination was found to contain more red cells than leucocytes, the latter amounting at most to one-fourth or one-fifth of the total number of cells.

- (3) 2.5 c.c. of washed red cells.
- (4) 5.0 c.c. of whole citrated blood.



The result of this experiment was as follows :

The monkey inoculated with whole citrated blood developed a typhus infection of medium severity after an incubation period of twelve days, whilst the animal which received the washed red cells had an abortive attack of five days' duration. On the other hand the inoculation of the cells from the leucocyte layer gave rise to a severe infection after a short incubation period of six days.

A second experiment was performed in a precisely similar manner with the blood taken on the thirteenth day of a severe case of typhus fever.

On this occasion the separation of the leucocytes from the other blood cells had been more successful, only a small proportion of red cells being present.

The results obtained were very similar to those of the first experiment. The inoculation of 5 c.c. citrated plasma produced a mild infection lasting seven days, and no reaction at all occurred in the monkey receiving the 3 c.c. washed erythrocytes. The animal inoculated, however, with the 1.0 c.mm. of leucocytes developed after seven days' incubation a very severe infection.

From these experiments Nicolle has concluded that the virus of typhus fever present in the circulation is contained within the leucocytes, the virulence of the plasma being due to the *débris* of leucocytes that it might contain.

This assertion has met with almost general acceptance, and is quoted in many text-books as an established fact (Dopter and Sacquépée, Kolle and Hetsch, etc.). The literature, however, contains certain criticisms of Nicolle's work. Kusama (1920), who also quotes Miyajima, contends, and not without reason, that the leucocyte material in these experiments would contain blood-platelets as well as leucocytes. They also point out that the possibility of the virus being free in the blood and of being brought down with the leucocytes and platelets by virtue of a similar specific gravity has not been disposed of. Da Rocha-Lima (1919) does not admit the validity of this latter objection, for, in his opinion, centrifugalisation during twelve minutes would not be sufficient to throw down an invisible virus. He argues, however, that 3.0 c.c. of red cells should certainly contain as many leucocytes as would be present in the 1 c.mm. of white cells in Nicolle's experiments, and should therefore have produced infection. It is quite obvious that only the complete separation of the leucocytes from the other blood-elements will be able to answer this question definitely.

#### ATTEMPTS TO CONFIRM NICOLLE'S ASSERTION BY THE PRODUCTION OF A CONSIDERABLE EXTRAVASATION OF LEUCOCYTES.

By the separation of the leucocytes from the other blood elements a concentration of the virus should readily be realised, but it is impossible to obtain leucocytes from the blood in any quantity, and exceedingly difficult to separate them from the other blood-cells. This difficulty was overcome by making use of the white cells which appear in the peritoneal exudate after injection of sterile broth.

Typhus-infected guinea-pigs at different periods after infection received 10 c.c. of sterile broth intraperitoneally. This injection was practised slowly in order to avoid traumatism of the capillaries and extravasation of blood. These animals were killed after intervals of from two to five hours. The abdominal wall was carefully dissected out and the exudate sucked up by means of a Pasteur pipette through a small opening made in the peritoneum. In most cases it was found that the cells present in the exudate consisted entirely of leucocytes (principally polymorphonuclears). In certain instances, however,

the fluid had a uniform rose tint, which was shown by microscopical examination to be due to the presence of numerous red cells.

It might possibly be argued as an objection to this method that the leucocytes contained in the peritoneal exudate will not necessarily carry the virus. This objection is a perfectly valid one, but we have, of course, to consider the time which has elapsed between the injection of the broth and the withdrawal of the exudate. These leucocytes come from the blood, passing out of the local capillaries in response to the presence of the broth in the peritoneal cavity. The first of these cells to appear in the exudate will therefore be those present in the blood-stream at the moment of the inoculation. These leucocytes should be virus-carrying ones. Later, as a result of the drain on the leucocytes of the circulating blood, those in the bone-marrow may be mobilised to make good the deficiency. Some of these in all possibility would appear in the exudate after a very short stay in the circulation and might not carry with them the typhus virus.

The proportion of these non-infective leucocytes in the peritoneal exudate would increase with the time that had been allowed to lapse between the inoculation of the broth and the withdrawal of the contents of the peritoneal cavity. However, it seems fairly safe to presume that the fluid withdrawn from the peritoneal cavity in the early stages of the formation of the exudate, two to five hours after the inoculation, would be composed, at any rate in greater part, of leucocytes present in the circulation at the moment of its inception. It should therefore contain a large proportion of virus-carrying cells.

Guinea-pigs were inoculated intraperitoneally with 4 to 5 c.c. of this exudate (containing in certain experiments over 58,000 leucocytes per c.mm.) or with the leucocytes obtained by centrifugalisation of a similar volume of fluid and emulsified in saline. In one experiment the leucocytes were extracted with distilled water.

*Results.*—In no case (four experiments) did the inoculation of leucocytes so obtained produce infection.

Similar negative results were obtained with the whole exudate when it contained leucocytes only (three experiments), whereas infection ensued in the animals inoculated with a blood-containing exudate (two experiments).

These experiments were controlled by the simultaneous inoculation of the blood or brain of the experimental animals, showing them to be infective in every case.

The guinea-pigs that did not react to the leucocytes or whole exudate inoculation were later tested for immunity.

The following temperature charts are typical of the results obtained in the above experiments.

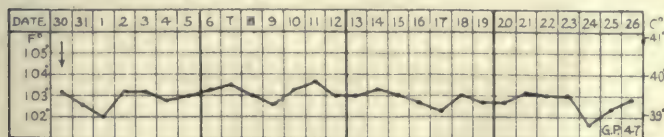


CHART 1.—Guinea-pig 47 inoculated with leucocytes obtained from 5 c.c. exudate and emulsified in saline.

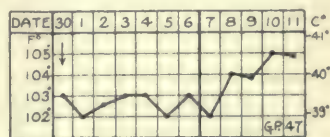


CHART 1A.—Guinea-pig 47 tested for immunity.



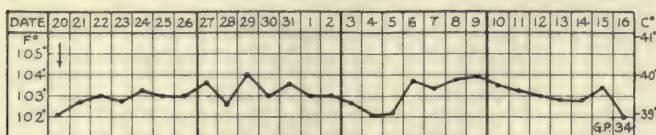


CHART 2.—Guinea-pig 34 inoculated with leucocytes of 4 c.c. exudate extracted with distilled water.

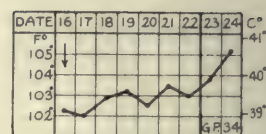


CHART 2A.—Guinea-pig 34 tested for immunity.

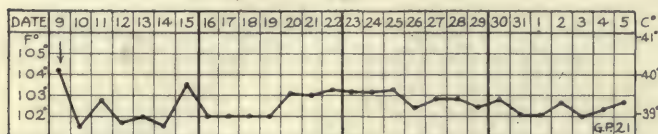


CHART 3.—Guinea-pig 21 inoculated with 4 c.c. whole exudate.

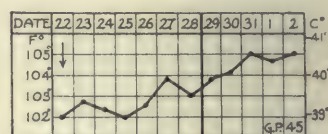


CHART 4.—Guinea-pig 45 inoculated with 5 c.c. of a pinkish exudate (containing about 30 per cent. of red cells).

#### ASSOCIATION OF THE VIRUS WITH THE PLATELETS.

At this juncture my attention was drawn to the work of Kusama (1920), who claims that there is an intimate connection between the virus of typhus and the blood plates.

This statement is based upon three experiments on Japanese monkeys. The inoculation of a quantity of platelets (free from leucocytes) obtained from 2.5 c.c. blood produced infection in the three cases, whereas the injection of the plasma obtained from a similar volume of blood and entirely freed from cells invariably gave negative results.

Experiments on guinea-pigs carried out on similar lines have given complete confirmation of the findings of this investigator.

The blood was received into an equal volume of a 2 per cent. citrate solution in saline containing 1 per cent. glucose (about 10 c.c. solution for each guinea-pig), centrifugalised 5 to 7 minutes at about 3000 revolutions per minute and then allowed to stand overnight in the cold room. The supernatant plasma, having a milky appearance, was then carefully removed and centrifugalised for forty-five minutes at about 6000 revolutions per minute. It is then found by microscopical examination that the yellowish supernatant fluid is cell free and the white sediment consists entirely of blood-plates.

*Results.*—Plasma carefully freed from cells by prolonged centrifugalisation (four experiments) proved non-infective even in large doses (5 c.c.), whereas the inoculation of platelets (six experiments) invariably produced infection even with so small a quantity as that obtained from 1.5 c.c. blood.

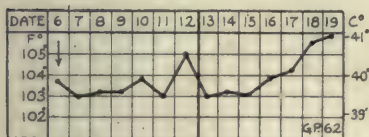


CHART 5.—Guinea-pig 62 inoculated with platelets from 4 c.c. blood. The cultures of the blood and brain of this animal were found to be sterile. Passed to Guinea-pigs 81, 82 and 83 with positive results.

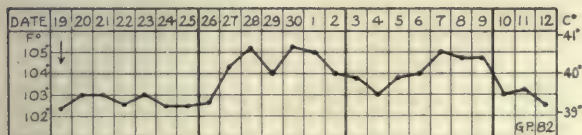


CHART 6.—Guinea-pig 82 inoculated with brain of Guinea-pig 62.

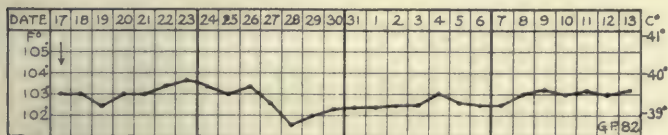


CHART 6A.—Guinea-pig 82 tested for immunity.

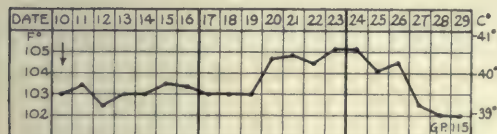


CHART 7.—Guinea-pig 115 inoculated with platelets from 1.5 c.c. blood.

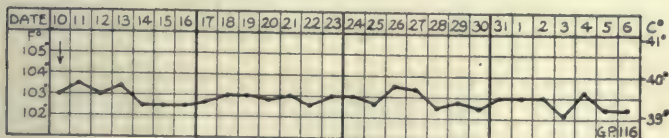


CHART 8.—Guinea-pig 116 inoculated with 5 c.c. plasma.

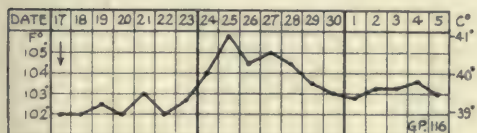


CHART 8A.—Guinea-pig 116 tested for immunity.



The leucocyte layer of centrifuged citrated blood can be shown to contain an abundance of platelets even when the blood has only been centrifuged during five minutes at a moderate speed. This fact may explain the findings of Nicolle, who, it will be remembered, worked with the leucocytic layer of blood which had been centrifuged a much longer time and must therefore have contained platelets in still greater quantity.

A high degree of concentration of the virus can thus be realised by the separation of the platelets by fractional centrifugation from large quantities of blood. With such a concentrated virus it has been found possible to infect guinea-pigs by the inoculation of a small volume in the anterior chamber of the eye and by intratesticular inoculation. In this latter case a marked atrophy of this organ has followed, which did not occur in the control animals inoculated with the same volume of platelets obtained from normal blood. It has also facilitated the experimental infection of lice in joint work with A. W. Bacot (Bacot and Ségal, 1922).

A field of experimental vaccination is also opened up and work along these lines has already commenced. Although it is too early to draw any conclusions, the result of the first experiment on guinea-pigs is encouraging.

#### CONCLUSIONS.

(1) Leucocytes obtained from peritoneal exudates of typhus-infected guinea-pigs do not carry the virus.

(2) The virus seems to be intimately connected with the platelets.

(3) By the separation of these elements from a large quantity of blood a high concentration of virus can be realised for experimental purposes.

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To many members of the staff of this Institute I am indebted for valuable help and advice, and I am especially grateful to Dr. Bedson for assistance in the preparation of this paper.

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# ANOXÆMIA AND THE INCREASED ELECTRICAL EXCITABILITY OF THE NEURO-MYONE.

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THE aim of the present work has been to determine the cause of the increased electrical excitability often manifested by the neuro-myone, *i. e.* the peripheral neuro-muscular mechanism. This increase has long been known to occur in idiopathic tetany and in tetania parathyreopriva; its cause in these conditions is disputed. That it is due to an action upon some peripheral part of the neuro-muscular system is shown by the cross-circulation experiments of MacCallum (1913) and his colleagues and by the work of Noël Paton, Findlay and Watson (1917).

## METHODS.

It will facilitate the consideration of the various theories if a brief description is first given of the methods adopted in this research.

1. The method used for measuring the strength of current causing contraction was that employed by Noël Paton, Findlay and Watson (1917), who give a criticism of its limits of accuracy. Figures for the anodal contractions are omitted because the records of von Frankl-Hochwart (1888) and Noël Paton, Findlay and Watson all show that these reactions are not constant.

2. Cats and dogs were used in all the experiments; anæsthesia was induced by chloroform and maintained by ether. A venous cannula was inserted in the left external jugular vein and was connected to a burette containing the fluid to be injected.

The electrical reactions were taken several times before the injection was commenced, and in the majority of cases varied little, if at all. Neither the anæsthetic nor the operation had any effect on the electrical reactions.

Arterial blood for examination was withdrawn from the right carotid artery into a 5 c.c. all-glass syringe rendered air-tight by a thin film of oil. When oxygen determinations were being made, blood was also taken from the right external jugular vein. The loss of blood thus caused was never found to produce any alteration in the electrical reactions.

3. Methods of estimating various changes in the blood:

A. *Alkaline reserve.*—The alkaline reserve was determined by the Van Slyke (1917) method.

B. *Hydrogen-ion concentration.*—This was estimated by the method described by Bayliss (1919–20).

C. *Oxygen-content of the blood.*—The oxygen content of arterial and venous bloods was determined by Barcroft's differential method (1914). Results are given in percentage saturation.

## PREVIOUS VIEWS.

Of the numerous theories put forward to explain increased electrical excitability (E.E.) of the neuro-myone, the principal are the following:

- A. The presence in the blood of excess of sodium.
- B. Decrease in the calcium content of the organism.
- C. A condition of acidosis.
- D. A condition of alkalosis.
- E. The presence of a toxic substance.



Of the toxic substances suggested, ammonia, histamine, guanidin and xanthin may be mentioned. Berkeley and Beebe (1909) suggested xanthin as a possible cause, but MacCallum and Voegtlin (1909) failed to confirm their hypothesis.

#### A. *The Effect of an Excess of Sodium in the Blood.*

Münzer (1893) stated that he could produce tetany by injection of various sodium salts. Rosenstern (1910) maintained that administration of common salt to "spasmophilic" children caused a recurrence of tetany, and Nothmann's work (1910) gave the same results. Parhon and Ureche (1907) had previously shown that injection of 1 per cent. sodium chloride into thyreo-parathyroidectomised animals caused a marked increase of symptoms. In all these cases where administration of sodium merely precipitated an attack in an organism with a spasmophilic diathesis, it is possible that this may have been due to disturbance of the sodium-magnesium ratio on which the irritability of a tissue to some extent depends (Loeb, 1900).

In order to test this possibility the following experiments were performed:

TABLE I.—0.9 per cent. NaCl was Injected.

Cat.	Weight in kg.	Amount of solu- tion in- jected in c.c.	Electrical reactions.				CO <sub>2</sub> capacity of plasma in volume per 100 c.c.		Temperature in degrees Fahr.	
			Before.		After.		Before.	After.	Before.	After.
3, female.	3.1	250	0.4	9.0	0.6	9.0	34.6	31.3	99.0	103.0
11, „	2.5	220	0.9	4.5	0.9	4.9	48.1	47.5	100.0	101.6
12, male	2.7	250	1.1	11.0	1.1	10.0	30.2	28.9	99.5	101.4

The only change to be noted was a rise in temperature during administration of the sodium solution. The alkaline reserve and electrical reactions were unchanged.

Further, Hastings, Murray and Murray (1921) found that in dogs with experimental pyloric obstruction there were signs of nervous hyper-irritability, but decrease in the concentration of sodium in two out of three cases.

It must therefore be concluded that excess of sodium can be nothing more than a predisposing factor in the production of an increase in the E.E.

#### B. *The Effect of Diminution of Calcium.*

Much of the evidence bearing on the influence of calcium on neuromyal excitability is contradictory, and, as Noël Paton, Findlay and Watson (1917) remark, "There is no conclusive evidence as regards the relationship of calcium to the condition of tetany." Some recent investigations merit attention.

MacCallum, Lintz, Vermilye, Leggett and Boas (1920) describe a case of pyloric obstruction accompanied by tetany in which there was a decrease in the calcium content of the serum. Hastings, Murray and Murray (1921) produced a pyloric obstruction in dogs, and using better methods of analysis than MacCallum and his colleagues, found a slight increase in the serum calcium. Harrop (1919) describes a case of adult tetany caused by injection of sodium bicarbonate but with no decrease in the calcium content of the blood. Grant and Goldman (1920-21) produced attacks of tetany by hyperpnœa, but found that the calcium content increased from 12.84 mg. to 13.44 mg. per 100 c.c. They suggest that the condition of the blood colloids was such that the calcium was present in saturated solution, and that any additional calcium was immediately precipitated or claimed by the lime-starved cells. They support this view by showing that the calcium content rose after injection of normal blood.

Howland and Marriott (1918) made analyses of the blood in cases of idiopathic tetany in children, found a distinct diminution in the calcium content, and obtained benefit by calcium therapy. Barach and Murray (1920), however, report a case of sprue complicated by tetany in which no relief was obtained by injection of calcium lactate.

The following experiments were done to throw some light on the influence of calcium on the E.E. They are divided into two groups:

- (1) To show the effect of calcium administration.
- (2) To determine if there were any changes in the calcium content of the serum when the E.E. had been altered by some other method.

(1) *Effect of calcium administration.*—1·9 per cent. calcium chloride was injected into the jugular vein with the following results:

TABLE II.—*Showing Effect of 1·9 %  $\text{CaCl}_2$ .*

Cat.	Weight in kg.	Amount of solution in c.c.	Electrical reactions.			
			Before.		After.	
			KCC.	KOC.	KCC.	KOC.
18, male	3·35	20·0	1·5	1·7	3·8	6·0
53, female	2·40	6·0	0·35	2·2	0·7	4·5
—	—	20·5	"	"	"	5·0
—	—	24·0	"	"	0·8	"
—	—	43·0	"	"	"	9·0
20, female	2·70	15·0	0·60	1·1	1·1	4·0

The results indicate quite clearly that calcium chloride injection has a sedative effect on the E.E. This may explain one of the beneficial effects of the therapeutic administration of calcium salts in cases of tetany as being due to its action on the central nervous system. But no evidence has yet been adduced that calcium has any real curative effect in idiopathic tetany or tetania parathyreopriva.

(2) *Changes in the calcium content of the serum after increase of E.E.*—10 c.c. of blood were withdrawn from the carotid artery before and after the increase in excitability. The calcium was estimated by a method used by Noël Paton, Findlay and Sharpe (1921), and I am indebted to Mr. J. Sharpe for the analyses.

TABLE III.—*Showing Effect of Increased E.E. on Ca-content of serum.*

No. of animal.	Weight in kg.	Solution used in c.c.	Electrical reactions.				Calcium oxide.	
			Before.		After.		Before.	After.
			KCC.	KOC.	KCC.	KOC.		
Cat 49, female.	2·9	Alcohol 10%; 18	0·6	6·0	0·5	1·5	0·019%	0·019%
Dog 4, , ,	7·4	$\text{Na}_2\text{CO}_3$ 1·9%; 200	1·2	8·0	1·0	5·5	0·017%	0·016%
Cat 73, male	2·8	HCl. N/20; 25	0·5	2·8	0·6	6·0	0·02%	0·02%

It will be seen that the calcium content remains practically unchanged both when the E.E. is raised and when it is lowered. We can therefore conclude that calcium deficiency is not the general causal factor in the change of E.E. observed under so many different conditions.



### c. *Effect of Acidosis.*

Several workers have put forward the view that an acidosis is the ultimate cause of increased nervous excitability.

Mathison (1910) found that the excitability of the skeletal-muscle nerve-centres of the spinal animal was increased by injection of acids. Later, Elias (1918), working on the effect of acids on the nervous system, stated that injection of relatively small quantities of acids produced a general hyperirritability of the peripheral nervous system. He concluded that in experimental acidosis the symptoms referable to the central nervous system are almost identical with the picture in tetany. Wilson, Stearns and Janney (1915) have shown that the symptoms of tetany can be relieved by acid injection. In parathyreoidectomised dogs Togawa (1919-20) found a condition of acidosis, while McCann (1918) observed a marked increase in the alkaline reserve in such animals. Hastings and Murray (1921) found the CO<sub>2</sub>-combining capacity of the plasma unchanged.

Experiments were carried out to determine the effect of acid on the E.E. of the neuro-myone; HCl (M/7), lactic acid (N/100) and phosphoric acid (N/10) were used.

TABLE IV.—*Showing Effect of Injection of Acids.*

No. of animal.	Weight in kg.	Solution injected.	Amount of solution in c.c.	Electrical reactions.				CO <sub>2</sub> capacity (V. Slyke) in c.c. per 100 c.c. of plasma.	
				Before.		After.		Before.	After.
				KCC.	KOC.	KCC.	KOC.		
2, male	2.8	HCl	170	0.8	2.5	1.0	8.0	32.5	18.9
8, "	2.4	"	250	0.4	2.0	0.7	9.0	35.1	11.2
9, female.	3.1	"	220	0.5	1.7	0.6	11.0	28.7	8.9
33, male	2.7	"	210	0.9	7.0	1.0	7.0	31.9	16.8
34, "	2.8	"	125	0.5	2.8	0.6	6.0	34.7	26.0
35, "	2.4	"	150	0.5	2.7	0.7	4.5	32.4	20.6
13, female.	2.3	Phos. Ac.	10	0.6	1.1	2.5	4.0	—	—
14, male	3.0	Lactic Ac.	200	0.7	3.5	0.9	6.5	32.9	21.1
15, female.	2.5	"	250	0.6	2.7	1.0	7.0	30.1	13.1
56, "	2.9	"	150	0.3	1.1	0.5	3.2	—	—

TABLE V.—*Showing Effect of M/7 HCl on pH.*

No. of cat.	Weight in kg.	Value for pH.			
		Previous to any injection.	After 50 c.c. injection.	After 100 c.c. injection.	After 150 c.c. injection.
33	2.7	7.4	7.4	7.4—	7.2+
34	2.8	7.4	7.4	7.4	—
35	2.4	7.4	7.4	7.4—	7.2

+ indicates a value slightly above figure indicated.

— indicates a value slightly below figure indicated.

Thus the E.E. is always lowered by the addition of acid unless the excitability is previously low as in Cat 33. Excitability that has been raised by administration of alkali is also quickly lowered by injection of acid. Usually there is a change in the values for the electrical reactions after 50 c.c. of acid (M/7 HCl) have been administered, but the extent of the change varies in individual cases. In no case was there any suggestion of tremors or convulsive

movements, as are commonly seen in parathyreoidectomised animals or in idiopathic tetany. Even the administration of phosphoric acid, which, according to Elias, is specially potent in producing hyperirritability, did not lead to any increase in excitability. There is a rapid decrease in the alkaline reserve immediately following the injection of acid, but there is no clear ratio between the change of the  $\text{CO}_2$ -combining power of the plasma and the amount of acid injected.

Barcroft (1914) has shown that blood becomes "meionectic" in presence of acid. Oxyhæmoglobin is more dissociated, so that the head of oxygen (*i. e.* the difference between the oxygen-saturations of arterial and venous bloods) becomes greater and the tissues receive a greater supply of oxygen.

A study of the results of injection of acids does not support the view that acid is a cause of hyperirritability of the neuro-muscular system. Far from causing an increased excitability it leads to a marked diminution. This probably explains the beneficial results obtained in cases of tetany treated by administration of acid.

#### D. *The Effect of Alkalosis.*

The effect of alkalis in raising the excitability of the neuro-myone has been noted for a long time.

L. Blum (1913) reported, in a patient suffering with diabetic coma, the occurrence of convulsions following the administration of sodium bicarbonate, and ceasing when the bicarbonate was stopped. Palmer and Van Slyke (1917) quote a communication by Tileston of the occurrence of severe convulsions in a case of Weil's disease following the intravenous injection of bicarbonate solution which produced a plasma  $\text{CO}_2$ -combining capacity of eighty volumes per cent. Harrop (1919) reports a case of tetany following the intravenous injection of bicarbonate without any diminution in the calcium concentration of the serum. Howland and Marriott (1918), although they support the calcium deficiency hypothesis as the cause of tetany, state that it is not unusual to see symptoms of tetany develop in cases of acidosis treated with sodium bicarbonate. P. S. Henderson (1920), however, who investigated the effect of sodium bicarbonate on children, could not confirm their results, finding that doses as high as 0.92 gm. per kilo body-weight per day had no influence on the E.E. even after twenty-three days, nor were any tetany symptoms produced. On the experimental side, Collip and Backus (1920-21) injected large doses of 5 per cent. sodium carbonate in distilled water intravenously into dogs, but in two cases only found the least sign of tetany. They did not, however, test the E.E.

In tetania parathyreopriva, the work of Wilson, Stearns and Janney (1915) pointed to the presence of an early alkalosis, as demonstrated by the sudden diminution in the urinary excretion of acids and the decrease in the hydrogen-ion concentration of the urine. Wilson, Stearns and Thurlow (1916) followed up this work by showing, from a study of the dissociation constant of oxyhæmoglobin and the alveolar  $\text{CO}_2$  pressure, that there was an increase in the alkalinity of the blood. They found that this alkalosis may be neutralised during tetany periods probably by the production of lactic acid in the muscles. Increase in the alkaline reserve of the blood-plasma was soon afterwards described by McCann (1918). Later experiments by Hastings and Murray (1921), who worked on dogs, failed to confirm these results. It has to be noted, however, that it is often very difficult to take the blood for examination during the period of alkalosis, which sometimes lasts for a very short time.

McCallum and Voegtlin (1909) have shown that when the pylorus is obstructed and the gastric juice with its hydrochloric acid constantly removed, there is produced an increase in the E.E. of nerves with spontaneous twitchings, ending in most cases in violent convulsions. Hastings, Murray and Murray (1921), in dogs with pyloric obstruction, found nervous hyperirritability, an increased  $\text{CO}_2$ -combining power of the plasma, and an insignificant rise in the pH.

Experiments were performed to determine the effect of alkalis on the E.E. of the neuro-myone.



(1) *Effect of sodium carbonate.*TABLE VI.—*Showing Effects of Injection of  $\text{Na}_2\text{CO}_3$  1.9%.*

No. of animal.	Weight in kg.	Amount of solution in c.c.	Electrical reactions.				CO <sub>2</sub> capacity of plasma.	
			Before.		After.		Before.	After.
			KCC.	KOC.	KCC.	KOC.		
Cat 1	3.2	240	1.0	7.0	1.1	1.0	31.0	67.1
" 4	2.1	100	1.6	11.0	1.0	1.4	28.9	61.6
" 5	2.7	200	1.2	10.0	1.0	1.3	31.9	68.3
" 6	2.7	150	0.8	6.0	0.7	1.1	33.5	69.9
" 7	2.9	250	0.5	4.8	0.3	0.8	30.8	72.5
" 12A	2.3	220	0.5	0.8	0.5	0.8	31.0	69.4
" 30	2.8	150	1.1	9.0	1.0	2.5	—	—
" 31	1.9	200	0.6	4.5	0.4	0.9	—	—
" 32	2.4	200	1.0	6.5	0.6	1.2	—	—
Dog 1	6.8	220	2.1	5.0	1.1	1.0	40.8	76.8
" 2	7.0	250	1.8	7.0	1.5	1.5	42.7	78.7

TABLE VII.—*Showing Effects of Injection of  $\text{Na}_2\text{CO}_3$  on Value of pH.*

No. of animal.	Weight in kg.	Value of pH.			
		Before injection.	After 50 c.c.	After 100 c.c.	After 150 c.c.
30	2.8	7.4	7.4+	7.7	8.0
31	1.9	7.4	7.7	8.0	9.0—
32	2.4	7.4	7.7—	8.0—	8.0+

— indicates a value slightly below figure indicated.

+ indicates a value slightly above figure indicated.

After sodium carbonate injections there was therefore an increase in the E.E. unless the excitability was previously high, when there was no change. There is no apparent relationship between the amount of alkali injected and the increase of excitability. Occasionally there were twitchings resembling those in parathyroidectomised animals.

The alkaline reserve of the plasma is increased by the injection of sodium carbonate, though there is no clear relationship between the change of the CO<sub>2</sub>-combining power of the plasma and the amount of alkali injected.

The value of the pH is often very much raised, the increase depending on the amount of carbonate injected (Table VII).

The following Table, VIII, gives the figures relating to the head of oxygen obtained from the oxygen-analysis of the blood.

TABLE VIII.—*Showing the Effect of  $\text{Na}_2\text{CO}_3$  on % Saturation of Oxygen.*

No. of animal.	Weight in kg.	Amount of solution in c.c.	% Saturation of oxygen.					
			Before.			After.		
			Art.	Ven.	Diff.	Art.	Ven.	Diff.
30, female.	2.8	150	94.0	58.6	35.4	88.1	69.8	18.3
31, "	1.9	200	94.8	52.0	42.8	80.1	72.6	7.5
32, male	2.4	200	92.4	47.8	44.6	84.5	70.9	13.6

It will be seen that there is always a reduction in the head of oxygen after injection of alkali, thus indicating a diminished supply of oxygen to the tissues.

(2) *Effect of sodium hydrate*.—In order to eliminate any effect of the carbon dioxide 2 per cent. sodium hydrate was injected, with the results detailed in Table IX.

TABLE IX.—*Showing Effect of NaOH 2 per cent.*

No. of cat.	Weight in kg.	Amount of solution in c.c.	Electrical reactions.				CO <sub>2</sub> comb. capacity in c.c. per 100 c.c. plasma.	
			Before.		After.		Before.	After.
			KCC.	KOC.	KCC.	KOC.		
13, female .	2.9 .	100 .	0.6 .	3.0 .	0.5 .	1.7 .	34.9 .	66.9
36, male .	1.6 .	25 .	0.6 .	6.0 .	0.4 .	1.5 .	29.1 .	60.3
37, „ .	2.7 .	150 .	1.4 .	9.0 .	1.0 .	2.6 .	31.1 .	87.9

Sodium hydrate has thus an action similar to the carbonate both on the E.E. and the alkaline reserve.

(3) *Effect of ammonia*.—Berkeley and Beebe (1909) found that muscular twitchings were produced by intravenous administration of ammonia. G. D. Cathcart (1916) also found tremors and convulsions following on intravenous injections of ammonium carbonate, persisting even when the animal was under full anæsthesia.

The following experiments were done to determine whether injections of ammonia had any effect on the E.E. of the neuro-myone.

TABLE X.—*Showing Effects of NH<sub>4</sub>OH. N/10.*

No. of cat.	Weight in kg.	Amount of solution used.	Electrical reactions.				CO <sub>2</sub> capacity of plasma (V. Slyke).	
			Before.		After.		Before.	After.
			KCC.	KOC.	KCC.	KOC.		
38 .	3.1 .	200 .	1.3 .	9.0 .	0.9 .	3.5 .	32.5 .	43.9
39 .	2.9 .	190 .	1.8 .	1.2 .	1.7 .	1.0 .	— .	—
67 .	2.9 .	200 .	1.2 .	7.5 .	0.4 .	1.5 .	31.9 .	51.1

These results indicate that ammonia causes an increase in the excitability as anticipated from the findings of Cathcart (1916).

We have seen that alkalis produce an increase in the E.E. of the neuro-myone unless that excitability is previously high, that the alkaline-reserve of the blood is increased while its hydrogen-ion concentration is diminished; further, the "head of oxygen" to the tissues is decreased.

M. H. Grant (1920-21), working on the isolated nerve muscle preparation of the frog, showed that the only pH capable of inducing increased excitability is 10.0, and, as this is in excess of what is found in the mammalian organism suffering with tetany, concluded that the H-ion concentration is not the chief factor in the production of increased E.E. The results of other experiments on the neuro-myone *in situ* tend to support this view. It will be shown later that alcohol, potassium cyanide and other substances have a distinct action in increasing E.E. without any action on the pH of the blood.

The following experiments further show that there may be an increase in the E.E. despite a fall in the alkaline-reserve.



TABLE XI.—*Showing Changes in the Alkaline Reserve accompanying Increase in E.E.*

No. of cat.	Weight in kg.	Substance injected.	Electrical reactions.				CO <sub>2</sub> comb. capacity in c.c. per 10 c.c. plasma.	
			Before.		After.		Before.	After.
			KCC.	KOC.	KCC.	KOC.		
49, female .	2.9 .	Ethyl Alc.	0.6 .	6.0 .	0.5 .	1.5 .	34.0 .	25.8
50, " .	3.1 .	" "	0.3 .	2.7 .	0.3 .	1.6 .	34.9 .	24.5
53, male .	3.0 .	Methyl "	0.8 .	10.0 .	0.35 .	2.2 .	38.7 .	27.8
62, female .	2.7 .	Pot. cyan.	0.6 .	5.5 .	0.6 .	2.2 .	33.9 .	18.4

Collip and Backus (1920-21) showed that one of the effects of hyperpnœa was a cramp-like contraction of the muscles, and that this was accompanied by a fall in the CO<sub>2</sub>-combining power of the plasma, a decreased acidity of the urine and a diuresis. They suggested that the muscle contraction was due to the alkalosis produced by the hyperpnœa. Grant and Goldman (1920-21) by hyperpnœa produced some symptoms of tetany, including an increased E.E. of the neuro-myone. The pH both of the blood and urine during the hyperpnœa indicated a blood-alkalosis, while the plasma CO<sub>2</sub> fell from an average figure of 59.5 vols. per cent. to one of 44.9 per cent. In a control experiment where washing out of CO<sub>2</sub> was prevented, no alkalosis nor any symptoms of tetany resulted. They suggest that alkalosis is the condition at the root of all the various forms of tetany. Hill and Flack (1910) also observed the spastic conditions of the muscles; in one case twitchings of the facial muscles occurred during hyperpnœa. When forcible breathing of oxygen was tried none of the twitchings or other uncomfortable signs of hyperpnœa were manifest.

#### EFFECT OF ANOXÆMIA.

The effects of acids and alkalis on the E.E. and on the supply of oxygen to the tissues suggest that anoxæmia may be the causal factor in the production of an increase in the E.E. of the neuro-myone. It has long been known that asphyxia raises the excitability of the central nervous system, and it seemed probable that lack of oxygen might produce a similar result in the peripheral parts of the neuro-muscular mechanism. Haldane (1919) has divided the causes of anoxæmia into—

- (1) Defective oxygen saturation of arterial blood.
- (2) Slowing of the circulation so that an excessive proportion of the oxygen is used up in the systemic capillaries.
- (3) Defective hæmoglobin content.
- (4) Alteration in the dissociation-curve of oxyhæmoglobin.

Experiments will now be detailed to show the effect on the E.E. of the neuro-myone of each of the above causes of an anoxæmia.

Defective saturation of the arterial blood with oxygen was produced by the induction of asphyxia, slowing of the circulation by diminution in the temperature, a defective proportion of available hæmoglobin by the production of an anæmia, and an alteration in the desaturation of oxyhæmoglobin by administration of potassium cyanide.

*A. Anoxæmia Produced by Asphyxia.*

The effects of asphyxia in raising the excitability of the central nervous system have long been known. Kaya and Starling (1909-10) found that asphyxiation of the spinal dog led to spasm of the whole body, and Mathison (1910) showed that this stimulation was due to lack of oxygen and not to the increase of carbon dioxide. Hooker, Wilson and Connett (1917) demonstrated the influence of anoxæmia in exciting nerve-centres.

Noël Paton, Findlay and Watson (1917) investigated the effects of asphyxia on the E.E. of the neuro-myone, and found that during brief periods of asphyxia there was little change. Adams and Morris (1920-21) have shown that there is practically no decrease in the head of oxygen until there is a marked degree of asphyxia. This might explain the absence of any change in the E.E. in the asphyxia experiments mentioned, for, as Haldane and Priestley (1905) have pointed out, there is a considerable margin as regards oxygen-supply.

The following experiments were undertaken to demonstrate the effect of deep asphyxia on the E.E. and determine the concurrent changes in the oxygen-content of arterial and venous bloods.

*Methods.*—Asphyxia was induced by making the animal breathe in a closed circuit in which there was placed a jar of soda-lime through which the air inspired had to pass. In this way the available oxygen was slowly used up and there was a gradual onset of anoxæmia without any accumulation of carbon dioxide.

TABLE XII.—*Showing Effects of Asphyxia.*

No. of cat.	Weight in kg.	Degree of asphyxia.	Electrical reactions.				Percentage oxygen content.					
			Before		After		Before.			After.		
			KCC.	KOC.	KCC.	KOC.	Art.	Ven.	Diff.	Art.	Ven.	Diff.
23, female.	2.6	+	0.6	7.0	0.6	7.5	95.4	63.7	31.7	74.2	33.8	40.4
31, „	2.4	++	0.6	8.5	0.6	8.0	91.0	57.5	33.5	64.2	30.6	33.6
23, „	2.6	+++	0.6	7.0	0.4	2.4	95.4	63.7	31.7	33.1	21.1	10.0
24, „	2.4	+++	1.1	7.5	0.5	1.7	96.0	58.3	37.7	29.9	17.2	12.7
25, male	2.8	+++	0.8	10.0	0.4	1.7	94.1	51.0	43.1	43.3	20.1	23.2
31, female.	2.4	+++	0.6	8.5	0.2	1.1	91.0	57.5	33.5	28.0	22.0	6.0

In the most marked stages of asphyxia there is a distinct increase in the E.E. of the neuro-myone associated with an equally marked diminution in the head of oxygen. When the asphyxia is not so complete the head of oxygen is still practically as great as normal and the E.E. remains unchanged.

*B. Effect of Anoxæmia Produced by Decrease of Temperature.*

Noël Paton, Findlay and Watson (1917), investigating the effect of variations of temperature on E.E., came to the conclusion that the E.E. of the nerve varies inversely with the temperature. These experiments were repeated with the results as given below:

*Methods.*—The electrical reactions on both hind legs were taken. One leg was then wrapped round with cloths soaked in hot water while the other was covered with small pieces of ice. After ten minutes the electrical reactions were again taken. The temperature of the legs was then brought back to normal and the femoral veins exposed, and blood taken from each. The warm and cold applications were again replaced, and after ten minutes blood was again withdrawn from each vein. The arterial oxygen content was estimated from blood drawn from the carotid artery.



TABLE XIII.—*Showing the Effect of Heat and Cold.*

No. of cat.	Weight in kg.	Electrical reactions.								Percent. oxygen content.			
		Normal.				Hot.		Cold.		Art.	Ven.		
		R. leg.		L. leg.		R. leg.	L. leg.	L. leg.	Norm.		Hot.	Cold.	
		KCC.	KOC.	KCC.	KOC.								
		KCC.	KOC.	KCC.	KOC.								
64, female.	2.4	0.4	7.0	0.5	7.5	0.5	7.0	0.2	2.6	93.6	61.0	69.9	52.3
65, „	2.9	0.5	6.0	0.4	6.0	0.5	5.5	0.4	1.5	95.0	65.4	73.7	52.1
68, male	2.8	0.5	2.2	0.5	2.8	0.5	2.4	0.5	1.1	94.4	57.9	66.5	49.2

The E.E. is increased by decrease in temperature, but the head of oxygen is apparently increased, blood from the vein on the cold side being more unsaturated than that from the hot side. Krogh (1918-19) has shown that the diffusion constant of oxygen through animal tissues increases with increasing temperature. This would explain the rise in the head of oxygen. In this connection there is an interesting observation by Lovatt Evans (1917) on the reduced sensitivity of the heart to adrenalin when the temperature is reduced. This phenomenon bears a strong resemblance to that found under the influence of oxygen-lack and seems to hint at the possibility of the same underlying cause.

Decrease of temperature, therefore, produces an increase in the E.E. of the neuro-myone. This can probably be explained by the diminished supply of oxygen to the tissues.

#### C. *Effect of Anoxæmia as Produced by Anæmia.*

MacCallum (1913) showed that ligation of an artery bringing about complete anæmia of the part induces for a time a marked increase in the E.E. of the neuro-myone, but that later the E.E. suddenly disappears as a result, probably, of death of the tissues. Noël Paton, Findlay and Watson (1917) repeated and confirmed these results.

The following experiments were performed in order to show the effect of anæmia on the E.E. The animal was bled from the carotid artery, and the electrical reactions taken and the oxygen content of the blood estimated before and half an hour after hæmorrhage.

TABLE XIV.—*Showing the Effect of Anæmia.*

No. of cat.	Weight in kg.	Total vol.* of blood.	C.c. of blood withdrawn.	E.R. before.		E.R. after.		
				KCC.	KOC.	KCC.	KOC.	
69 .	2.1 .	115.5 .	60 .	0.9 .	11.0 .	0.4 .	2.1 .	
70 .	2.8 .	154.0 .	50 .	1.1 .	8.5 .	0.4 .	1.8 .	
71 .	3.2 .	176.0 .	50 .	1.0 .	9.0 .	0.8 .	2.5 .	
% Sat. of O <sub>2</sub> before.				% Sat. of O <sub>2</sub> after.			Hæmoglobin per cent.	
No. of cat.								
	Art.	Ven.	Diff.	Art.	Ven.	Diff.	Before.	After.
69 .	92.5 .	60.1 .	32.4 .	51.7 .	37.9 .	13.8 .	100 .	63.0 .
70 .	96.0 .	64.6 .	31.4 .	69.9 .	60.6 .	9.3 .	100 .	71.0 .
71 .	94.4 .	61.4 .	33.0 .	74.1 .	59.1 .	15.0 .	100 .	76.0 .

\* Estimated on the assumption that the volume of the cat's blood is equivalent to 5.5 per cent. of the body-weight.

The figures show that the E.E. is increased by the production of an anæmia. In every case there occurred marked general tremors of greater or less intensity. The head of oxygen is diminished.

D. *Effect of Anoxæmia as Produced by Cyanides.*

It has been demonstrated by many that the oxygen consumption of tissues is markedly diminished by cyanide administration. Lovatt Evans (1919-20) has shown that soaking in cyanide has almost the same effect on the frog's sartorius as keeping it in an atmosphere of nitrogen, and concludes that cyanides exert their effects entirely by reason of the oxygen lack they produce; in muscle they do not affect the contractile, but the recovery process.

Potassium cyanide made up to M/100 in normal saline was injected intravenously into cats. If we assume that the blood weighs about 5·5 per cent. of the total body-weight, then the administration of 1 c.c. of the above dilution per kilo body-weight means a concentration in the blood of a 0·00012 M. solution of potassium cyanide. The concentrations of cyanide used here would certainly cause a definite though temporary depression in the amount of tissue oxidation.

TABLE XV.—*Showing Effect of Cyanide.*

No. of cat.	Weight in kg.	Amount of solu- tion injected in c.c.	Electrical reactions.				Comb. CO <sub>2</sub> per 100 c.c. plasma (V. Slyke).	
			Before.		After.		Before.	After.
			KCC.	KOC.	KCC.	KOC.		
60	3·1	7·0	1·1	9·0	1·0	1·9	—	—
61	3·4	4·0	0·9	10·0	0·6	5·0	30·9	28·4
62	2·9	9·0	0·6	5·5	0·6	2·2	33·9	18·4

These results indicate that the E.E. is increased by the administration of cyanide. In all three experiments general convulsions of central origin were produced, and in Cat 62 tremors of the abdominal muscles were particularly noticeable.

There is a fall in the CO<sub>2</sub> content of the plasma, *i.e.* an "acidosis" as some (Evans and others) have suggested, and also probably in part a result of the initial increase in depth and rate of respiration.

Administration of cyanide thus induces an increased E.E. of the neuro-myone as well as other symptoms of profound oxygen lack. This lends further support to the view that anoxæmia is the essential factor in the production of such an increase in the E.E.

THE INFLUENCE OF ALCOHOL ON ELECTRICAL EXCITABILITY AND THE HEAD OF OXYGEN.

If anoxæmia is the causal factor in the production of increased E.E., we should find the latter state after administration of alcohol, if it were shown that alcohol depresses the oxygen usage of the tissues, as most narcotics do.

Ethyl alcohol diluted with nine times its volume of 1 per cent. sodium chloride solution was injected into the jugular vein. In one experiment



methyl alcohol gave the same results except that convulsions and very marked salivation and sickness was induced.

*Small doses.*—Schweisheimer (1912–13) found in one case of drunkenness in man that the blood contained 0.153 per cent. of alcohol, and in another instance, when the intoxication was more pronounced, 0.227 per cent. Mellanby (1919) found that in a dog, when the alcohol of the blood reached about 354 c.mm. per 100 grm. blood, there appeared signs of intoxication, and when the concentration reached 468 c.mm. per 100 grm. the dog was profoundly intoxicated. In the first four experiments an amount of alcohol was injected to produce a concentration in the blood of 350 to 450 c.mm. per 100 grm. blood, *i.e.* a concentration one would expect to find in a case of fairly marked alcoholic intoxication.

TABLE XVI.—*Showing the Effect of Small Doses of Alcohol.*

No. of cat.	Weight in kg.	Amount of alcohol solution 10%.	Concentration of alcohol* c.mm. per 100 grm. blood.	Electrical reactions.				CO <sub>2</sub> combining capacity in c.c. per 100 c.c. blood.	
				Before.		After.		Before.	After.
				KCC.	KOC.	KCC.	KOC.		
44	3.0	6.0	363	0.4	4.0	0.4	3.8	34.6	21.9
45	2.7	5.0	403	1.2	8.0	1.0	7.5	31.8	22.7
46	2.3	5.0	395	0.6	5.5	0.6	6.5	33.6	22.7
47	2.8	7.0	454	0.9	7.0	0.7	5.5	35.4	18.8

\* The figures in this column were estimated on the assumption that the blood was 5.5 per cent. of the total body-weight.

With such doses there is not produced any marked change in E.E.

*Large doses.*—When larger doses of alcohol are injected so that the concentration in the blood exceeds 1 per cent. there is produced a marked increase in E.E., as will be seen from the accompanying table:

TABLE XVII.—*Showing Effect of Large Doses of Alcohol.*

No. of cat.	Weight in kg.	Amount of alcohol solution (10%) in c.c.	Concentration of alcohol* c.mm. per 100 grm. blood.	Electrical reactions.				CO <sub>2</sub> combining capacity in c.c. per 100 c.c. plasma.	
				Before.		After.		Before.	After.
				KCC.	KOC.	KCC.	KOC.		
48	2.4	10	757	1.4	8.0	1.3	3.5	37.4	20.9
49	2.9	18	1130	0.6	6.0	0.5	1.5	34.8	18.2
50	3.1	12	706	0.3	2.7	0.3	1.6	35.3	23.7
51	2.5	10	724	1.0	9.0	0.8	2.6	35.9	26.5
53†	3.0	12	727	0.8	10.0	0.3	2.2	38.3	28.9

\* The figures in this column were estimated on the assumption that the blood was 5.5 per cent. of the total body-weight.

† Methyl alcohol was injected in this case.

The alkaline reserve is rather lower than with the smaller doses. The increase of CH is now no longer able to neutralise the reduced action of the blood catalase as can be seen from a study of the following table:

TABLE XVIII.—*Showing the Fall in the Head of Oxygen after Large Doses of Alcohol.*

No. of cat.	Weight in kg.	Amount of alcohol solution (10%) in c.c.	Conctr. of alcohol em. per grm. blood.	% Saturation of oxygen.					
				Before.			After.		
				Art.	Ven.	Diff.	Art.	Ven.	Diff.
51	2.5	10	724	89.4	60.7	28.7	80.4	69.8	10.6
76	1.9	10	952	92.1	61.9	30.2	74.3	60.9	7.4
77	2.8	12	779	90.8	50.8	40.0	72.6	60.9	11.7

This fall in the head of oxygen is probably in great part due to the marked slowing and at times complete inhibition of respiration. This phenomenon was observed by Hooker (1917).

It is probable that alcohol in such excessive doses acts as a protoplasmic poison, lowering the sensitivity of the respiratory centre especially, and by this means as well as by its action on the tissue catalase preventing the utilisation of oxygen by the tissues. This lends support to the view previously put forward that increased E.E. is dependent on a deficient supply of oxygen.

#### THE INFLUENCE OF HISTAMINE ON ELECTRICAL EXCITABILITY AND THE HEAD OF OXYGEN.

Dale and Dixon (1909-10), in an exhaustive study of the pharmacology of histamine, showed that it acted on the bronchial musculature and pulmonary arteries. Its action when applied directly to striped muscle was practically *nil*. Biedl (1916) suggested that tetany might be due to the presence of this substance in the blood. Noël Paton, Findlay and Watson (1917) administered histamine to a few animals but observed none of the symptoms of tetany. They did not test the E.E. of the neuro-myone.

Anderes and Cloetta (1916), in a study of the effect of histamine on cats, state that it produced a rapid decrease in oxygen-absorption.

The following experiments were done to determine (1) whether administration of histamine caused a decrease in the head of oxygen, and (2) if this was the case, whether the E.E. of the neuro-myone was affected. 0.5 mg. dissolved in 1 c.c. of saline was injected into the jugular vein.

In cat 49 as the first dose produced but little effect another 0.5 mg. was given twenty minutes later.

TABLE XIX.—*Showing Effects of Histamine.*

No. of animal.	Weight in kg.	Electrical reactions.				O <sub>2</sub> content.			O <sub>2</sub> content.		
		Before.		After.		Before.			After.		
		KCC.	KOC.	KCC.	KOC.	Art.	Ven.	Diff.	Art.	Ven.	Diff.
47, female	2.6	1.1	11.0	0.4	3.5	94.6	59.3	35.3	77.9	62.7	15.2
48, male	3.0	0.9	7.5	0.3	2.8	86.9	51.0	35.9	71.8	49.9	21.9
49, female	2.9	0.6	7.0	0.5	5.0	93.7	59.9	33.8	81.2	56.7	24.5
49, after 2nd dose	2.9	0.6	7.0	0.4	3.0	„	„	„	65.4	50.1	15.3

Histamine produces an increased E.E. of the neuro-myone as well as a diminution in the head of oxygen. Tremors were not seen in any of the three experiments performed, thus confirming the observations of Noël Paton,



Findlay and Watson mentioned above. The result obtained with Cat 49 bears out the statement of Dale and Dixon that the action of histamine on the respiratory system of cats is variable.

The action of histamine on the E.E. of the neuro-myone and on the head of oxygen lends support to the view that the excitability is dependent on the oxygen supply.

#### THE INFLUENCE OF GUANIDIN.

Noël Paton, Findlay and Watson put forward the suggestion that guanidin was the essential cause of the symptoms of tetania parathyreopriva. They point out, however, that on the administration of guanidin the E.E. is often not at once altered, and suggest that there is "an optimum concentration for causing an increase in the reaction of the peripheral mechanism," and that at greater concentrations a curare action manifests itself. This conclusion is supported by the work of Meighan (1917).

The following experiments (Table XX) were done to determine whether the administration of guanidin had any effect on the oxygen supply to the tissues. Guanidin-hydrochloride was the salt used, dissolved in 20 c.c. saline and injected into the jugular vein.

TABLE XX.—*Showing Effect of Guanidin.*

No. of cat.	Weight in kg.	Dose per kilo in gm.	Electrical reactions.				% Saturation of oxygen.					
			Before.		After.		Before.			After.		
			KCC.	KOC.	KCC.	KOC.	Art.	Ven.	Diff.	Art.	Ven.	Diff.
67	2.7	0.074	0.5	2.7	0.4	2.4	89.5	53.9	35.6	83.7	51.1	32.6
68	2.2	0.140	0.4	7.0	0.2	6.0	93.1	64.6	28.5	87.7	63.1	24.6
69	3.1	0.160	0.9	8.0	0.5	7.5	94.5	69.1	25.4	89.3	56.1	33.2
70	2.3	0.210	0.4	5.0	0.6	6.0	90.6	72.2	18.4	94.1	71.9	22.2

As will be seen from the above table, guanidin seems to have had very little effect on the "head of oxygen." But in none of the four experiments was there any marked increase in E.E., nor were any tremors observed. Accordingly one cannot come to any conclusions as to whether the action of guanidin in producing an increased E.E. is due to a diminished supply of oxygen to the tissues or to some specific action of its own.

In cases of parathyroidectomy, it has been noted by Wilson, Stearns and Janney (1915) that the blood became pleonectic. This would suggest that, when guanidin does cause symptoms similar to those of parathyroidectomy, including almost identical disturbances in metabolism, it might also render the blood pleonectic.

#### SUMMARY AND CONCLUSIONS.

(1) Excess of sodium does not of itself produce an increase in the E.E. of the neuro-myone.

(2) Whilst injection of calcium salts has a sedative effect on the neuromyal excitability, the present work lends no support to the view that diminution of calcium is the underlying cause of increased excitability. The amount of calcium in the blood shows no change when the excitability is increased by certain methods.

(3) The administration of acids lowers the E.E. and renders the blood mesonectic.

(4) The administration of alkalis increases the E.E., produces an alkalosis, and lowers the oxygen-supply of the tissues.

(5) Asphyxia, lowering of the temperature and anæmia each causes an increase in excitability and a diminished supply of oxygen to the tissues.

(6) Cyanides raise the E.E. and lower the oxygen-supply.

(7) Small doses of alcohol have practically no effect either on the E.E. or on the oxygen supply, whilst large doses cause a rise in the former and a fall in the latter. The alkaline reserve is diminished.

(8) Histamine produces a rise in the E.E. as well as an anoxæmic condition.

(9) No further light has been thrown on the action of guanidin, but there is reason to believe that it also may act through a diminished oxygen-supply to the tissues.

A general survey of these results justifies the following conclusions.

(1) An increase in the E.E. occurs independently of change in the alkaline reserve.

(2) Any experimental method whereby an anoxæmia is produced (a diminution of alkalis, cyanides, histamine or asphyxia) causes an increase in the E.E. Acids lower the E.E.

(3) Any increase in the E.E. is accompanied by a diminished supply of oxygen to the tissues.

(4) One must therefore conclude that anoxæmia is the essential condition underlying an increased E.E. of the neuro-myone.

I must express my deepest thanks to Prof. D. Noël Paton for his very helpful criticism throughout the course of the work. I am indebted to Mr. Graham, Senior Laboratory Attendant at the Institute of Physiology, for assistance in the experimental work.

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*The late MR. ARTHUR W. BACOT,  
Entomologist to the Lister Institute, London.*

# THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY

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## IN MEMORIAM.

ARTHUR W. BACOT, F.E.S.,

Entomologist to the Lister Institute, London.

*Born April 28th, 1866, Died April 12th, 1922.*

By the death of Arthur Bacot, Entomologist to the Lister Institute, from typhus fever contracted in the course of experimental research into the ætiology of this disease in Cairo, medical entomology loses an investigator whose unique attainments had gained him world-wide repute. It was only in January of this year that Bacot and his colleague Arkwright proceeded to Cairo at the request of the Egyptian Government to continue researches into the nature and life-history of the typhus virus which in London were not fully satisfied by the employment of passage virus only. So far as technical methods went, the transference to Cairo meant to them simply a change of laboratory. They were accommodated at the Hygienic Institute in rooms placed at their disposal by the Director, Dr. Charles Todd, an old colleague of both. Precisely how Bacot, and shortly afterwards Arkwright, now happily convalescent, became infected, will perhaps never be known. In the course of their work they had found, in contrast to their own previous experience in London (see Atkin and Bacot, *Brit. J. Exper. Pathol.*, not yet published) and to that of Rocha-Lima, but in harmony with statements of Nicolle, that the excreta of typhus-infected lice were capable of conveying the disease to guinea-pigs, and it is just possible that infection arose from this source, especially as their work involved much handling of louse-boxes charged with virulent faecal material. Both experts, however, knew well the risks they ran, and while providing against accidents so far as humanly possible, were not deterred from the pursuit of knowledge by the chance of some unlooked-for avenue of infection. To the long list of workers whose labours to solve the mystery of typhus fever have claimed the last penalty must now be added the name of Arthur Bacot.

At his death Bacot had all but completed his 56th year, and it was only 11 years ago that the opportunity came to him to bring his great knowledge of insect lore and bionomics—the fruit of a boyhood's hobby but the scientific passion of maturer years—to bear on the elucidation of vital problems of epidemiology. He was educated at the Birkbeck School, on leaving which he became a clerk in a city office, and there he remained till he was 45 years of



age. In his hours of leisure, however, he turned to his insect studies, and much of his early work in entomology was concerned with breeding experiments in Lepidoptera with special reference to analysis of Mendelian phenomena. Bacot was most successful as a breeder of insects for statistical analysis on Mendelian lines, and he was equally successful when at a later period in his career he undertook the study of blood-sucking insects in connection with human disease. At practically all times or at shortest notice Bacot could supply for his own experiments or those of others, lice, bugs or mosquitoes fed on his own person. Bacot rapidly became a lepidopterist of repute, and many of his contributions to entomology between the years 1890-1910 will be found in various entomological journals such as *The Entomologist's Record* and the *Proceedings of the Entomological Society of London*, of which he became a Fellow in 1901. In 1910 Bacot was requested by the Plague Research Commission to undertake a study of the bionomics of fleas, a subject on which the precise information available was but meagre. Further knowledge was indispensable to the elucidation of the rôle played by the rat flea in the transmission of plague from rat to man. Bacot undertook the work with enthusiasm, and by the provision of an assistant it was made possible for him to organise and supervise the multifarious experiments connected with the problem without totally severing his connection with the office stool. Within 18 months the ground had been largely covered, and the result was a compendium of knowledge on the life-history and bionomics of rat fleas which for all time must form the hunting ground of workers in this sphere. The experiments in connection with this work were carried out at Loughton, Essex, where Bacot lived, and the collated results are embodied in a lengthy monograph contributed to the *Journal of Hygiene*.

This research and its outcome so completely displayed Bacot's talents for undertaking and bringing to a conclusion a piece of systematic work under rigidly controlled conditions, that it became obvious that his attainments must be given scope for their fullest development by the opportunity of uninterrupted devotion to the medical aspects of entomology. He was consequently invited, in 1911, by the Governing Body of the Lister Institute, to accept the position of Entomologist to the Institute—a new post specially created for him. Henceforward he could devote himself solely to the development of his specialty and its application to current medical and epidemiological problems. To take an effective part in research requiring the services of variously trained experts, necessitates a very careful dovetailing of the various units concerned. To fit himself for such collaborative work Bacot was not long in familiarising himself with technical procedures hitherto new to him, such as sectioning and staining of insect tissues, and such was his handiness that he became most adept in this difficult art. A working knowledge of bacteriological technique also did not come amiss to him, so that in a remarkably short space of time he was able to undertake some complete problem unassisted or take an effective share in the problem of a team.

The exact mechanism of transmission of plague from rat to rat or rat to man by means of the rat flea was not clear.

The matter was clarified by a fascinating piece of work carried out by Bacot in conjunction with C. J. Martin. It was found that in the stomach of

the flea (*Xenopsylla cheopis* and *Ceratophyllus fasciatus*) which has sucked blood containing plague bacilli, the latter multiply rapidly and may form veritable masses of culture which fill up the proventriculus and even extend forward through the gullet. Fleas showing marked blocking of the proventriculus due to this cause are not prevented from sucking blood, as the pump is situated in the pharynx—they suffer indeed from thirst and bite with abnormal avidity—but the result of the pumping is that the already contaminated œsophagus is simply distended. At the conclusion of the pumping process some of the blood, carrying plague bacilli with it, is forced back into the wound. Success in transmitting plague experimentally to rats by plague-infected fleas was found to be largely, if not wholly, conditioned by a blocked proventriculus. Fleas in this condition are in danger of drying up if the temperature is high and the degree of saturation low, and it is probable that certain features of plague incidence in various parts of India, which appear to be correlated with local meteorological conditions, are in reality primarily dependent on the response of the blocked flea to such climatic variations.

Among other researches which occupied Bacot's attention at this time should be noted his work on the persistence of bacteria in pupæ and imagines of *Musca domestica* raised from larvæ which had been allowed to feed on a test bacillus. In the case of *Musca domestica* certain organisms, such as *B. pyocyaneus* supplied in the larval stage, were able to survive the cataclysm of the metamorphosis and appear in the pupæ and imagines. In the case of fleas, however, Bacot was unable to show that similar organisms could survive the pupal stage, and flea larvæ (*C. fasciatus*), taken from the bodies of mice dead from bubonic plague, and which had had opportunity to feed on fæcal material contaminated with *B. pestis*, showed little evidence of the presence of this organism after dissection and staining of the stomach contents. The conditions in the larval interior of the flea do not appear to be favourable to multiplication of *B. pestis*, just as the larval interior of the fly does not appear to be favourable to the persistence of pathogenic bacterial species, such as *B. typhosus* (Ledingham, Tebbutt, etc.).

In July, 1914, Bacot proceeded to Sierra Leone to take part, as Entomologist, in an investigation into yellow fever, his services being placed at the disposal of the Colonial Office.

The outbreak of war interfered with the carrying out of the scheme of work proposed, but Bacot stayed in West Africa for a year studying the bionomics of *Stegomyia fasciata*, and later published a very complete monograph on the subject.

He returned to this country in October, 1915, and soon found himself immersed in studies on the bionomics of lice with a view to devising efficient methods of sterilising clothing and preventing louse-borne infection at the Front. Bacot's experiments on pediculicides were invariably carried out on himself under conditions similar to those met with in the field. He accepted the position of Honorary Adviser to the War Office on entomological questions, and henceforward he was constantly consulted on matters relating to insecticides.

Opportunity to bring his knowledge of lice and their habits to bear on a problem of great medical and military importance came in December, 1917,



when a Trench Fever Committee formed by the Director-General of the Army Medical Service was constituted under the Chairmanship of Sir David Bruce. Bacot had control of the entomological side of the inquiry, was responsible for the supply of infected lice, and superintended their feeding on trench fever patients and the collection of the louse excreta for further experiment.

In the course of this work Arkwright, Bacot and Duncan confirmed the observation of Töpfer that curious small bodies resembling *Rickettsia prowazeki* in typhus-infected lice were present in the gut lumen of lice fed on trench fever patients. These bodies did not appear in the louse till a period of eight or twelve days had elapsed after a feed on a trench fever patient, and it was only then that such lice were capable of again infecting man.

Trench fever disappeared with the war, and with it the opportunity of continuing research on the *Rickettsia* bodies associated with it. So many analogies, however, existed between trench fever and typhus fever, alike in connection with louse-transmission generally and association with *Rickettsia* bodies in particular, that Bacot and his colleagues resorted naturally to typhus research so far as this could be adequately prosecuted in London with passage virus. In 1918, and again in 1920, virus had been obtained from Ireland with which work was undertaken. In the latter year a further opportunity came to Bacot to study this disease when he joined, as Entomologist, the Commission appointed by the League of Red Cross Societies to prosecute typhus research in Poland under the guidance of Drs. Wolbach and Todd. While in Warsaw Bacot was alive to the possibility of finding evidence of the existence of trench or volhynian fever, and such evidence came in a surprising manner. While feeding on his own person lice collected from a public bath-house Bacot developed a sharp fever which necessitated his removal to hospital under suspicion of typhus fever.

During his stay in hospital he continued to nourish these lice and in due course they developed extracellular *Rickettsia* forms exactly similar to those met with in trench fever lice at home. For some months after recovery he apparently still harboured the trench fever virus in his blood and was able to infect otherwise clean lice by feeding them on himself. The proof that what he had suffered from in Warsaw was trench fever and not typhus was complete enough—a full account of his illness with experimental data appeared in the *British Medical Journal*—and his death from typhus fever almost exactly two years later is further proof, if proof be needed.

The work of the Warsaw expedition has recently been published in book form, and in it the claim of *Rickettsia prowazeki* to represent the actual virus of typhus fever is strongly urged. The chain of evidence, however, was not complete and it was therefore with lively anticipations that Bacot and Arkwright proceeded early in January of this year to Cairo to prosecute further researches into the ætiology of typhus.

Since his visit to Poland, Bacot had been able to practise the intrarectal infection of lice on the lines of Weigl's method, and had succeeded in keeping lice alive for fairly long periods on an intrarectal diet of human blood. By the aid of this new weapon Bacot and Ségal were able to watch the development of *Rickettsia* in lice thus infected, and to complete a chain of evidence by successfully infecting guinea-pigs with such lice, and thereafter demonstrating the

occurrence of *Rickettsia* in lice inoculated intrarectally with the blood of these infected guinea-pigs. These most recent experiments by Bacot in conjunction with Ségal and Atkin are in course of publication in this Journal.

No sketch of Bacot's life-work would be complete without some reference to his personal charm, his single-minded devotion to science, and his unflinching desire to be helpful to others. Colleague, acquaintance or casual visitor alike experienced at his hands that old-world courtesy now perhaps rarely seen among us. He leaves indeed a pleasant memory, and his works will live after him.

J. C. G. LEDINGHAM.

## SCIENTIFIC CONTRIBUTIONS.

1893-1910.

### GENERAL ENTOMOLOGY.

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- "Variation of the Larvæ of *Saturnia carpini*," 4, 1893.
- "Perils of Egg Life," 6, 1895.
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- "The Relationship of *Endromis versicolor* to the Sphingides," 7, 1896.
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- "Notes on the Early Stages of *Enodia hyperanthus*," 8, 1896.
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- "On the Caudal Horn of *Agdistis*," 9, 1897.
- "Notes on Hybrid *Smerinthus populi-ocellatus*," 9, 1897, continuation in 10, 1898.
- "The Larvæ of certain Geometrids," 10, 1898.
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- "Position of Egg laid by *Sphinx ligustri*," 10, 1898.
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 "Notes on the Life-history of *Ctenonympha corinna*," 15, 1903.  
 "Notes on the Life-history of *Melitæ didyma*," 15, 1903.  
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THE INFECTION OF LICE (*PEDICULUS HUMANUS*) WITH  
*RICKETTSIA PROWAZEKI* BY THE INJECTION PER  
 RECTUM OF THE BLOOD PLATELETS OF  
 TYPHUS-INFECTED GUINEA-PIGS AND  
 THE RE-INFECTION OF OTHER  
 GUINEA-PIGS FROM THESE  
 LICE.

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FOLLOWING Weigl's (1920) plan of feeding lice by rectal injections (Bacot, 1922), female specimens of *Pediculus humanus* were given an infecting meal of platelet material (Ségal, 1922) obtained by fractional centrifugation of the blood of typhus-infected guinea-pigs (G.P.). These lice were thereafter fed on defibrinated normal human blood by rectal injection, or for a portion of their infective period (in the case of Batches 32 and 33) naturally by allowing them to bite healthy *Macacus* monkeys. Although the insects fed greedily on the monkey they died within three to four days, apparently because of their inability to digest its blood.

The lice were incubated at 32° C. (90° F.) and fed twice daily for the major portion of the period following their infecting injection, but for some 40 or 56 hours in each week they were kept at room temperature (about 18° C., = 65° F.) and received only one meal during this period.

A number of the lice died showing a heavy bacterial contamination of the alimentary tract; in most *if not all* of the cases the growth of bacteria was almost certainly the actual cause of death.

From the fact that the cases of bacterial contamination were very capriciously distributed among the five batches of infected lice dealt with in this paper, and that the insects contaminated died within four to six days of the infecting meal; there seems no doubt that the source of the contamination was the platelet material\* rather than the normal human blood on which they were fed subsequently to the infecting meal. This conclusion is supported by work now in progress.

The development of *Rickettsia prowazeki* was ascertained by dissecting out the guts of specimens which died and making smears of the teased-up guts, or by making smears of recently voided excreta, the former being the more certain of the two methods.

\* Accidents which occurred during the preparation of the platelet material in certain cases render it possible that sterility was not absolute.



In a few instances *Rickettsia* bodies were present in the smears which showed bacterial contamination, but in most cases there had either been insufficient time for them to develop, or else they had been overwhelmed by the more rapidly growing bacteria.

### Batch 31.

19 females of *Pediculus humanus* were injected with platelet material obtained from G.P. 72 (Chart 1).

5 specimens	died on the 3rd day.	All negative.
5	" " " 4th "	2 of which showed a few thread or short rod forms of <i>R. prowazeki</i> .
3	" " " 5th "	1 showed doubtful but no definite <i>Rickettsia</i> bodies.
		1 showed a few short rod forms (+).
		1 showed a few thread forms (++)
2	" " " 6th "	1 doubtfully infected.
		1 showed thread forms (+).



CHART 1.

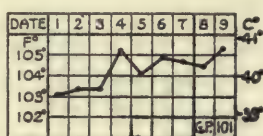


CHART 2.

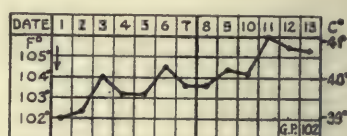


CHART 3.

1 specimen	killed on the 9th day.	Showed short rod and thread forms (++++).
		An emulsion of this gut was injected into G.P. 101 (see Chart 2).
1	" " " 10th "	Showed short rod and thread forms (++++).
		An emulsion of this gut was injected into G.P. 102 (see Chart 3).
		An emulsion of its ovaries was injected into G.P. 103 (see Chart 4).
1	" " " 10th "	Fixed for sectioning (unexamined).
1	" " " 10th "	Showed short rod and thread forms (++++).

*Summary.*—No contaminating bacteria. 6 out of 18 specimens examined showed *R. prowazeki*. Guinea-pigs 101, 102 and 103 were injected with emulsions from organs of the infected lice.

*Results of injections of louse material into guinea-pigs.*—G.P. 101 (Chart 2) injected intra-peritoneally with emulsion of gut. This G.P. was passaged by an injection of 2 c.c. of its blood into G.P. 110 and by an injection of an emulsion of  $\frac{1}{3}$  of its brain into G.P. 111. G.P. 110 and 111 showed a positive reaction. G.P. 110 was tested for immunity within sixteen days of its infec-

tion by the injection of an emulsion of  $\frac{1}{3}$  of the brain of an infected guinea-pig and gave a positive result. It is possible that this test was performed before immunity had been established, but it has been recorded by Da Rocha-Lima that guinea-pigs that have reacted to an infection of typhus virus have again responded to a second injection.

G.P. 102 (Chart 3) received an injection of an emulsion of the ovaries from the same louse the gut of which was used to infect G.P. 103. This G.P. was passaged by the injection of an emulsion of  $\frac{1}{3}$  of its brain into G.P. 122 and 123, both of which showed a positive reaction.

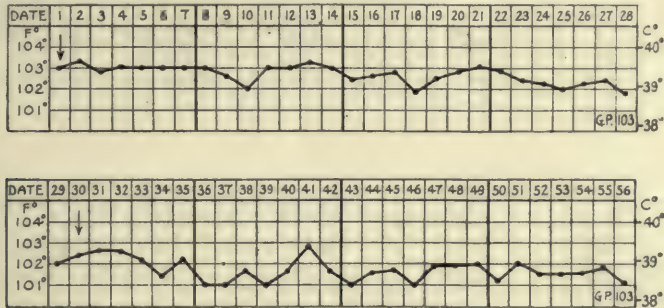


CHART 4.

G.P. 103 (Chart 4) received an injection of the gut of the same louse the ovaries of which were used to infect G.P. 102. G.P. 103 did not react to the injection, and was subsequently tested for immunity on the 30th day by an injection of brain emulsion from an infected G.P. As it failed to react to this we conclude that the animal was naturally immune.

#### Batch 32.

21 females of *P. humanus* were injected with platelet material from typhus-infected G.P. 84.

3	specimens	died within	3 hours.	Possibly owing to injury during manipulation.
3	"	"	20 "	Cause not ascertained; probably injury or bacterial contamination of gut.
2	"	"	3 days.	1 showed a few <i>Rickettsia</i> forms.
				1 negative.
2	"	"	4 "	The smear preparations were inadvertently destroyed.
5	"	"	5 "	3 showed bacterial contamination.
				1 showed <i>Rickettsia</i> infection (+).
				1 showed <i>Rickettsia</i> infection (++).

Smears made from the excreta of the 6 living specimens on the 7th day showed that some of the insects were heavily infected by *R. prowazeki*. These 6 lice were fed on a healthy *Macacus* monkey from this date until their death, the two last being killed only when they were too feeble to feed.



- 1 specimen died on the 9th day. Showed a heavy infection of small forms of *R. prowazeki* (+ + + +).
- 1 " (dying) was killed on the 10th day. Showed a heavy infection of threads and small forms of *R. prowazeki* (+ + + + +).
- An emulsion of the gut of this louse was injected into G.P. 121 (Chart 5).

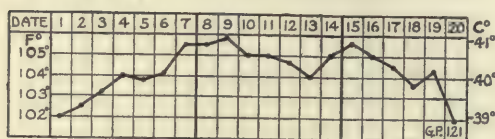


CHART 5.

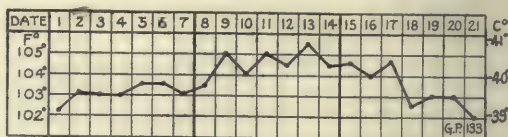


CHART 6.

- 1 specimen died on the 11th day. } Fixed for sections (unexamined).
- 1 " " " 12th " }
- 1 " killed " 13th " Both heavily infected with *R. prowazeki*,
- 1 " " " 13th " mostly small forms (+ + + + +). Gut emulsions of one injected into G.P. 133 (Chart 6), and of the other into G.P. 134 (Chart 7).

*Summary.*—9 died from unascertained cause, 3 died showing bacterial contamination, 9 died or were killed showing infection with *R. prowazeki*. Guinea-pigs 121, 133 and 134 were injected with emulsions of guts of these lice.

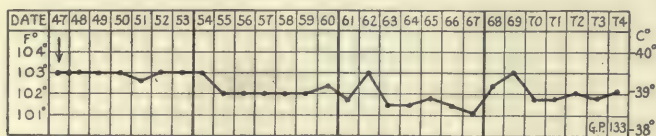


CHART 6A.

*Results of injections of louse material into guinea-pigs.*—G.P. 121 (Chart 5). Emulsion of gut intra-peritoneally. Died on the 20th day after injection.

G.P. 133 (Chart 6). Emulsion of gut intra-peritoneally. This G.P. was tested for immunity by the injection of  $\frac{1}{3}$  brains on the 47th day after the first injection (Chart 6A).

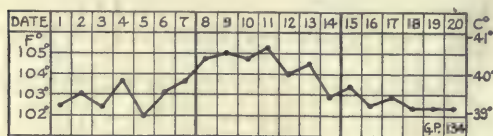


CHART 7.

G.P. 134. Emulsion of gut subcutaneously (Chart 7). This pig was tested for immunity by the injection of  $\frac{1}{3}$  brain on the 35th day after the first injection, but the animal died on the 14th day without reaction from unascertained cause.

## Batch 33.

12 females of *P. humanus* were injected with platelet material from typhus-infected G.P. 87. The preliminary wash in 2 per cent. lysol was omitted in the case of this batch. It is very doubtful, however, if the heavy incidence of bacterial contamination is attributable to this cause.

2 specimens died shortly after injection. From the second day after the infecting meal the remaining 10 were fed naturally by allowing them to suck blood from a healthy *Macacus rhesus* monkey. Within 5 days all had died, showing heavy bacterial contamination of their guts. In every case a few poorly stained *Rickettsia* forms were observed.

## Batch 34.

18 females of *P. humanus* were injected with platelet material from typhus-infected Guinea-pigs 137 and 146.

3					specimens died within 2 days. Two were examined; both showed heavy bacterial contamination of the gut.
6	"	"	"	3	" All showed heavy bacterial contamination of the gut.
2	"	"	"	4	" Both showed heavy bacterial contamination of the gut.
2	"	"	"	5	" 1 showed heavy bacterial contamination of the gut. 1 showed a rather indefinite infection, probably of <i>R. prowazeki</i> .
1	specimen killed on the 7th day.				Showed slight infection of <i>R. prowazeki</i> (+). An emulsion of the gut was infected into G.P. 183 (Chart 8).
1	" died "		9th	"	Showing heavy bacterial contamination of gut and also poorly stained <i>R. prowazeki</i> (+ +).
1	" " "		10th	"	Showing slight infection with <i>R. prowazeki</i> (+).
1	" (dying) was killed on 14th day.				Showing very heavy infection with <i>R. prowazeki</i> (+ + + +).
1	" was killed on 14th day.				Was fixed for sectioning (not yet examined).

**Summary.**—2 died unexamined; 11 died showing heavy bacterial contamination of gut; 1 died showing heavy bacterial contamination and also infection with *R. prowazeki*; 1 died showing possible infection of *R. prowazeki*; 3 showed definite infection of *R. prowazeki*.

**Result of infecting G.P. 183 with emulsion of gut.**—G.P. 183 was injected with an emulsion of the gut of a louse of Batch 34 (Chart 8). Material from this G.P. (183) was passaged (injection of an emulsion of  $\frac{1}{3}$  of its brain) to Guinea-pigs 201 and 202 (Charts 9 and 10).

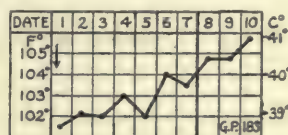


CHART 8.



G.P. 202 failed to react within 35 days and died before it could be tested for immunity.

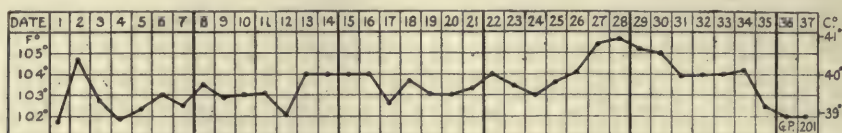


CHART 9.

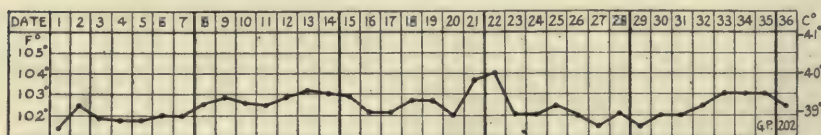


CHART 10.

### Batch 35.

17 females of *P. humanus* were injected with platelet material from typhus-infected G.P. 141.

6 specimens died within 24 hours.	} All showed a more or less heavy bacterial contamination of the gut.
1 specimen " 36 "	
1 " killed on the 4th day.	

Shown an indefinite picture; very slight if any development of *R. prowazeki*.  
An emulsion of the gut was injected into G.P. 182 (Chart 11).

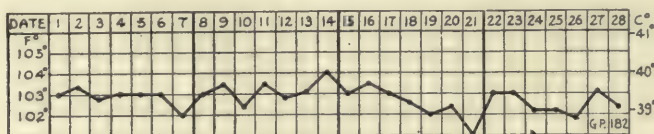


CHART 11.

1 specimen (dying) killed on 7th day.	} Showed (+ + +) infection of the gut-cells with thread and bacillary forms of <i>R. prowazeki</i> .
1 " killed on the 8th "	
1 " " " 11th "	

Shown threads and small bacillary forms (+ + + +) of *R. prowazeki*.  
An emulsion of the gut was injected into G.P. 191 (Chart 12).  
Shown minute bacillary forms of *R. prowazeki* (+ + + +).  
An emulsion of gut (+ + + +) was used to infect another batch of lice, and an emulsion of the ovary (+) was used to infect G.P. 198 (Chart 13).

1 specimen (dying) killed on 12th day. Showed minute bacillary forms (+ + + +) of *R. prowazeki*.

An emulsion of the gut (+ + + +) was injected into G.P. 199 (Chart 14).

An emulsion of the ovary (+) into G.P. 198.

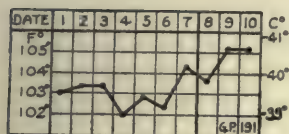


CHART 12.

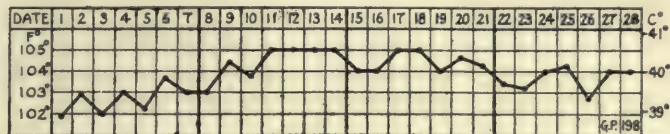


CHART 13.

1 specimen died on the 13th day. Showed minute bacillary forms of *R. prowazeki* (+ + + +).

1 „ killed „ 13th „ Fixed for sectioning (unexamined).

2 specimens died „ 14th „ Showed small bacillary forms of *R. prowazeki* (+ + + +) and few threads.

1 specimen killed „ 14th „ Showed small bacillary and short rod forms of *R. prowazeki* (+ + + +).

*Summary.*—1 unexamined; 7 died showing bacterial contamination; 1 indefinite picture; 8 showed more or less heavy infection of *R. prowazeki*.

Guinea-pigs 182, 191 and 198 were injected with emulsions of organs of lice of Batch 35.

*Results of infecting guinea-pigs and lice with emulsions of organs from Batch 35.*—

G.P. 182. Infected with emulsion of gut (Chart 11) failed to react.

G.P. 191. Emulsion of gut (Chart 12).

G.P. 198. Emulsion of the ovaries of two lice injected on successive days (Chart 13).

G.P. 199. Emulsion of gut injected into testicle: died on the 12th day (Chart 14).

21 females of *Pediculus humanus* were injected with an emulsion of gut of a louse of Batch 35 and defibrinated human blood (Batch 36).

Smears of lice of this batch showed a more or less heavy infection of *R. prowazeki*. There were no cases of bacterial contamination.

Judging by the previous experience of one of the authors, the time occupied by the development of *R. prowazeki* in the louse when the platelets were used as the infecting material was only about  $\frac{1}{2}$  to  $\frac{2}{3}$  that required for an equivalent development when the lice were fed on an infected monkey or when whole blood of infected guinea-pigs was injected.

This is, no doubt, due to the greater concentration of the organism in the platelet material than in whole blood, and the time needed for lice to acquire a

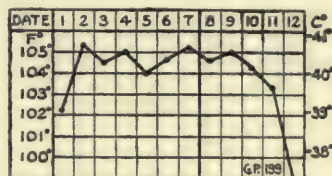


CHART 14.



heavy infection can be still further reduced by using as infecting material an emulsion of the gut of a heavily infected louse.

Apparently in whole blood the number of infecting units is so few that there are not enough gut-cells infected to enable them to be readily detected even at the end of their necessary incubation period. Later a secondary massive infection of the gut-cells takes place, probably as a result of the rupture of those first infected and the discharge of their contents into the lumen of the gut.

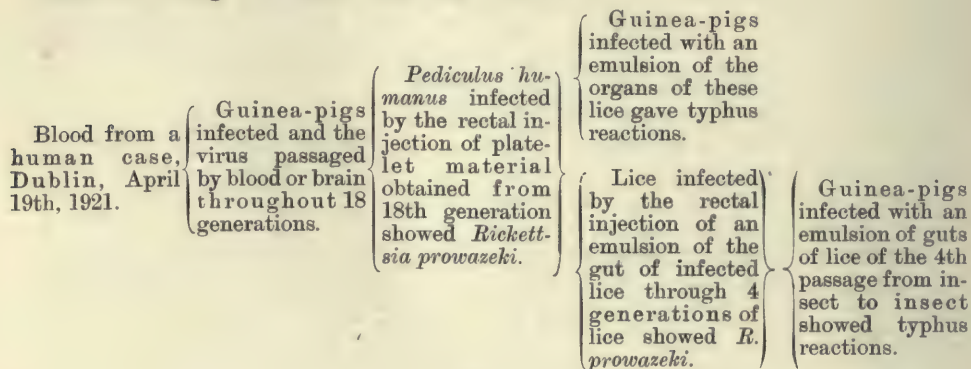
#### CONCLUSIONS.

(1) The injection of lice (*Pediculus humanus*) with a concentrated emulsion of platelets obtained by fractional centrifugalizations of the blood of a typhus-infected guinea-pig affords a sure and quick method of obtaining the development of *Rickettsia prowazeki* in these insects.

(2) The lice thus infected can be used to convey typhus fever to fresh guinea-pigs.

(3) The parallel development of typhus virus and *Rickettsia prowazeki* in successively passaged guinea-pigs is demonstrated and also in lice infected from guinea-pigs after 23 blood or brain passages.

The following succession has been obtained :



We desire to express our thanks to our colleagues Drs. Ledingham, Arkwright and Atkin for advice and assistance. Our grateful acknowledgments are also due to Dr. Atkin for supplying the virus, which he had already passaged through 8 generations of guinea-pigs before we started with the strain.

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# VARIATIONS IN THE DIASTATIC POWER OF THE URINE IN RELATION TO ITS REACTION, WITH A SUGGESTED METHOD FOR THE ESTIMATION OF THE DIASTASE CONTENT.

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SINCE the discovery by Wohlgemuth (1908) of a diastatic enzyme in the urine, its estimation has taken a very important place among the chemical aids to diagnosis, and has proved itself to be of great value in the detection of certain diseases, more especially in pancreatic and renal lesions.

In certain cases of nephritis (Wohlgemuth, 1909) the diastatic power of the urine is subnormal, whereas in lesions of the pancreas (Wohlgemuth, 1910) it is very much higher than normal. It has been said to be high in eclampsia (Wallis, 1920), and the writer has demonstrated that acute rickets is accompanied by a very high diastatic power. From what has been said it becomes obvious that the reaction is of considerable importance, both from the clinical and from the research aspects. Probably the diseases mentioned above vary the urinary diastase content by acting in one of two ways: (a) by altering the production of diastase (pancreatitis), or (b) by interfering with the excretion into the urine (nephritis). Now if the reaction is to be of value in the diagnosis of these diseases, then the above two conditions, and these alone, must be allowed to operate. It is the object of this paper to show that other purely chemical factors can influence the reaction, and if these are not eliminated, the value of the test is materially diminished.

By far the most important of these factors is the hydrogen ion concentration, and, to a lesser extent, the salt concentration of the urine (Evans, 1912). In the original technique (Corbett, 1913; Stocks, 1916) no account is taken of either, though they may vary to such a degree as seriously to interfere with the estimation of the diastase.

## THE REACTION OF THE URINE IN RELATION TO DIASTATIC POWER.

Many workers have called attention to the importance of the effect of hydrogen ion concentration on enzyme action, and research on this problem has proved that for each enzyme there is a definite optimum pH, and that this optimum reaction differs for different enzymes.

Michaelis and Peckstein (1914) showed that salivary diastase formed



compounds with salts, such as chlorides, sulphates, nitrates and phosphates, that each of the resulting compounds was amylolytic, and that each had an optimum pH. The phosphate, sulphate and chloride compounds worked best at pH 6.1, 6.1 and 6.7 respectively, and it was found that the chloride compound could be replaced by excess of phosphate, and similarly the other compounds were interchangeable. The metallic radicles had little or no effect. In common with all enzymes any alteration of hydrogen ion concentration, either above or below the optimum, led to a decrease in its amylolytic action.

These researches suggested an inquiry into the effect of reaction upon urinary diastase, and the first investigation carried out was the fixing of the optimum pH. Since buffer solutions of varying reaction are so easily made with mixtures of phosphates, it was decided to determine the optimum pH of the phosphate diastase compound in urine. This was done by taking a quantity of urine and diluting it 1 in 5 with buffer solutions of varying pH. The diastatic power of these mixtures was then determined in the way described below for the new technique. A number of urines were treated in this manner, and the results, calculated as described on p. 135, are seen in Table I. As in the case of salivary diastase, 6.1 appears to be the optimum pH for the phosphate diastase compound of the urine.

TABLE I.—*Optimum pH of Diastatic Power of Urine.*

Urine no.	Diastatic power at pH.							
	4.	4.5	5.	5.5.	6.1.	6.5.	7.0.	7.5.
1 .	10	25	25	28	33	28	20	10
2 .	0	0	10	20	20	10	6.6	0
3 .	0	0	6.6	10	28	25	20	10
4 .	0	6.6	10	20	28	25	20	0
5 .	6.6	10	20	25	28	25	20	10
6 .	10	20	20	25	33	25	20	15
7 .	0	6.6	10	20	20	10	6.6	0
8 .	25	33	66	70	80	50	25	10
9 .	0	10	20	28	33	28	20	10
10 .	0	6.6	10	20	28	20	20	10
11 .	10	20	20	28	33	28	28	20
12 .	0	0	10	20	28	20	20	10
13 .	10	10	20	20	28	20	20	10
14 .	20	28	28	33	50	33	33	20
15 .	0	10	20	20	33	20	10	0
16 .	10	10	15	20	25	20	20	15

Now, suppose that three urines, all containing the same quantity of diastase, but with reactions of pH 4.5, 6.1 and 7.0, are examined for diastase content by means of the old technique, all three will give different values; the middle one, being at the optimum pH, will give a much higher value than the other two, and thus it is obvious that all urines must be brought to the optimum reaction before being tested for diastatic power.

After a great many trials of different methods the following was found to be by far the easiest and best.

#### NEW METHOD PROPOSED.

*Solutions required.*—(1) A 0·2 per cent. starch solution. This is best made up by adding the weighed quantity of starch pinch by pinch to the requisite volume of distilled water, and stirring until an even suspension is obtained. The mixture is then slowly brought to the boil, stirring all the time. After boiling for a short time the solution becomes opalescent, when it is cooled and the volume is made up to the correct amount. By this means no troublesome gelatinous masses form and the solution is quite uniform. Preferably this solution should be made up fresh each day and should never be kept more than a few days.

(2) Phosphate buffer solution. This is obtained by mixing 15 c.c. of Sörensen's solution A with 85 c.c. of solution B.

Solution A is made by dissolving 11·876 gm. of  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$  in one litre of boiled distilled water, the solution being kept in a paraffin-coated bottle.

Solution B is made by dissolving 9·078 gm. of  $\text{KH}_2\text{PO}_4$  in 1 litre of boiled distilled water, and is stored in a paraffin-coated bottle.

The resulting solution should have a pH of 6·1.

In the whole of this investigation the buffer solutions have been checked by the hydrogen electrode.

*Method.*—1·5 c.c. of urine are added to 6 c.c. of the buffer solution. The resulting solution is well shaken in order to ensure uniform distribution. A series of test-tubes are then put up, each being filled as follows:

	Test-tube No.											
	1.	2.	3.	4.	5.	.	.	.	.	.	12.	
0·2 per cent. starch solution	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.	.	.	.	.	.	1 c.c.	
Buffered urine	1·5 c.c.	1 c.c.	0·5 c.c.	0·45 c.c.	0·4 c.c.	.	.	.	.	.	0·05 c.c.	
Distilled water	0·5 c.c.	1 c.c.	1·5 c.c.	1·55 c.c.	1·6 c.c.	.	.	.	.	.	1·95 c.c.	

Short tubes are used ( $\frac{1}{2} \times 4''$ ), as with these there is less liability for drops of the various ingredients to stick to the sides of the tube, and, by dropping down when the iodine is added, spoil the end-point. The urine and the fractions of a c.c. of water are added first, followed by the starch solution. It now remains to add rapidly 1 c.c. of water to each tube except No. 1. The sides of the tube are deliberately washed down into the bottom with this distilled water. By adding the solutions in the above order all the tubes get about the same digestion time. The tubes are incubated for half an hour at 37° C., cooled, and N/50 iodine added. As small a quantity as will just give a faint colour is used, excess making the end-point much more obscure.

*Calculation of results.*—Suppose the tube just not showing a mauve tint, *i. e.* the one where the starch was just digested, contained 0·5 c.c. of the diluted urine. Therefore 0·5 c.c. of diluted urine, or 0·1 of the undiluted urine, just digests 1 c.c. of 0·2 per cent. starch, or 2 c.c. of 0·1 per cent. starch. Since the number of Wohlgemuth's units is given by the number of



c.c. of 0.1 per cent. starch solution digested by 1 c.c. of urine, it is obvious that in the above case 20 units of diastase were present. 0.2 per cent. starch solution is used instead of 0.1 per cent. in order that the total volume may be kept to 3 c.c. as in the original technique. If greater volumes are used the end-point becomes indistinct.

Distilled water has been substituted for saline usually used as the fluid for making up to the constant volume. It is difficult to see the reason for using saline, and the writer has found that it tends to obscure the end-point.

In Table II will be seen the comparison of the old and modified technique on 50 urines. It will be seen that the statements already made are fully borne out, and that unless the reaction of the urine is pH 6.1 the diastase is hampered, and an incorrect estimate is obtained by the old technique.

TABLE II.—*Diastatic Power of Urine according to Old and New Method.*

Urine no.	Ordinary method.	New method.	pH of urine.	Urine no.	Ordinary method.	New method.	pH of urine.
1	28	33	6.5	26	25	50	4.5
2	5	10	4.5	27	2	6.6	4.5
3	20	30	7	28	6.6	10	5.5
4	5	10	4.5	29	2	10	4.5
5	5	10	5	30	10	25	4.5
6	2	6.6	4.5	31	2	10	5
7	28	30	7	32	2	10	5
8	28	30	7	33	20	66	4.5
9	6.6	10	6.8	34	2	6.6	5
10	10	22	6.8	35	20	40	4.5
11	10	6.6	6.8	36	2	10	5
12	10	20	7	37	22	30	5
13	5	6.6	4.2	38	22	25	5.5
14	2	10	4.2	39	6.6	10	6.8
15	150	150	6	40	20	33	4.5
16	10	10	6	41	6.6	10	5.5
17	10	10	6	42	25	30	5
18	150	150	6	43	25	30	5.5
19	10	10	6	44	5	20	8
20	20	25	5.5	45	2	6.6	7
21	10	10	6	46	2	6.6	7
22	10	10	6	47	2	6.6	8
23	25	28	6.8	48	2	10	7.5
24	10	10	6	49	2	10	8
25	10	20	4.8	50	0	25	8.5

It has been stated that ammoniacal decomposition has no effect on the diastase reaction. The last seven urines in Table II were ammoniacal, and it will be seen that, owing to their high alkalinity (pH 7 to pH 8.5), they gave abnormally low readings by the old method, but quite normal figures by the new. This obviously might lead to errors in diagnosis. Also, if urine which contains the slightest deposit be allowed to stand over-night the diastase sinks to the bottom, being absorbed to the surface of the deposit. A series of urines

were left to sediment over-night, and a diastase reaction was carried out on the supernatant part of the liquid, and on the sediment.

From Table III it will be seen that there is very much more diastase in the bottom of the jars than the top. That this was only due to an adsorption on to the deposit was shown by centrifuging clear urine for a long time, when no sedimentation of the diastase could be detected.

TABLE III.—*Absorption of Diastase by Urinary Deposits.*

Urine no.	Before centrifugalisation.	After centrifugalisation.	
		Supernatant fluid.	Fluid from bottom of tube.
1. Marked deposit .	33	2	25
2. " " .	10	25	28
3. " " .	20	0	10
4. " " .	28	10	25
5. " " .	50	0	60
6. No deposit .	20	20	20
7. " " .	10	10	10
8. " " .	6.6	6.6	6.6

#### SUMMARY.

(1) The original technique for the diastase reaction is criticised because it takes no account of the varying reaction of normal urine.

(2) It is proved that the optimum reaction for urinary diastase in the presence of phosphate was pH 6.1, as in the case of the salivary diastase compound.

(3) A modified technique is described whereby the urine is diluted with a phosphate buffer solution, thus bringing all urines to the optimum pH before its starch-digesting power is tested.

(4) Ammoniacal decomposition, by making the urine more alkaline, decreases the diastatic power as determined by the old method, but has no effect on the method suggested.

(5) Diastase tends to cling to urinary deposits, hence all urines should be well shaken before the estimation is performed.

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## AN INVESTIGATION INTO THE ÆTIOLOGY OF DENTAL CARIES. I: THE NATURE OF THE DESTRUCTIVE AGENT AND THE PRODUCTION OF ARTIFICIAL CARIES.

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CONSIDERING the wide-spread and almost universal distribution of dental caries, it is not a little surprising that we have no definite knowledge of the causal agent or agents. Numerous theories have been propounded in explanation, details of which are to be found in the various monographs which have appeared from time to time. It is therefore not proposed to deal with these views individually here. Perhaps the parasitic theory, or, as it is more aptly expressed by Miller (1890), the chemo-parasitic theory, has received most support.

The chemo-parasitic theory of dental caries may be regarded as having originated with Miller's brilliant researches. The purely inflammatory view is of course much older. Miller regarded acids produced by the fermentative action of bacteria as the primary factor in dental decay. He, however, lived in advance of his time; his bacteriological technique was exact, but bacteriological knowledge was not sufficiently advanced to profit by his researches or to identify the organisms he found in the lesions. Many of the bacteria observed by him failed to grow on the media at his disposal, so that much of his study was purely morphological. He definitely showed that mouth bacteria, by their action on carbohydrates, could produce sufficient acid to injure teeth. Since Miller's time most of the work seems to have consisted in enumerating the various bacteria found in the mouth and in dental caries—researches which might be more aptly described as investigations into the bacterial associations of dental caries. Miller enumerated a very large number of bacteria and classified them according as to whether he was able to grow them or not. Goadby (1903) in his valuable monograph described some eighteen types, which he subdivided into acid-producing types and dentine liquefiers. Kligler (1915) identified ten different groups. Howe and Hatch (1917), although no exact details or actual figures are given, consider that two types of bacteria, which they group together under the name "Moro-Tissier," are most commonly associated with dental caries.

## NATURE OF THE DESTRUCTIVE AGENT.

*Decalcification.*

In taking up this research on dental caries we were of opinion that if bacteria played an important rôle, then it was necessary to find micro-organisms which were capable of softening enamel, which process histopathological research has shown to be the initial lesion in caries. For the present, of course, we do not intend to deal with any of the predisposing causes, although the part played by structural deformities, whatever be their origin, cannot be denied. We have already noted that Miller considered that the production of acid by bacteria was the initial process. We therefore have endeavoured to discover a bacterium or group of bacteria present in dental caries which can produce sufficient acid to decalcify enamel and dentine. Once decalcification has been produced, the path is left open for almost any micro-organism to invade the dentine.

It is reasonable to suppose that the bacteria which produce large quantities of acid will be able to live in relatively high concentrations of acid. Therefore, bacteria which are able to decalcify enamel must be able to live in a very acid medium. This is briefly the thesis on which we have based this research and the basis of our technique to discover the bacterium.

The first procedure was to determine what degree of acidity is required to decalcify tooth enamel. This can be arrived at in two ways—experimentally and theoretically.

Experimentally the simplest procedure is to place normal teeth (*i. e.* teeth showing no caries) into solutions of different degrees of acidity. In our experiment we used acid nutrient broths of different pH values. The acid broths varied from pH 5 to pH 1, and the teeth were left in for thirty-four weeks. The teeth had previously been sterilised in the broth by autoclaving. At the end of the period the degree of whitening or opacity of the enamel was noted. It was found that no change was to be seen in those teeth placed in broths of pH values higher than 4, and only such a minute trace as to be negligible in pH 4 (Fig. 1).

The theoretical determination is much more difficult, as the formulæ have all been constructed to deal with elements in solution. Dealing with the carbonate radicle, it is possible to arrive at an approximate value by the calculation of the pH value of a saturated solution of carbonic anhydride. A saturated solution such as might occur in the mouth would give a solution corresponding to about N/1000.

From the formula  $\sqrt{K_1 \times 1}$ , where  $K_1$  = the dissociation constant, we get a pH value of 4.8 for such a solution.

The chemical affinity of acids in equivalent solutions is proportional to the dissociation constants, therefore we can assume that the acidic values must be in excess of this pH value before the carbonic oxide can be displaced from a compound, as it is reasonable to suppose that teeth are not affected by the  $\text{CO}_2$  of the saliva.

*Technique of Isolation.*

Carious teeth were obtained from the Dental Out-Patient Department and from two private practices in order to include as many different types of



mouth conditions as possible. A tooth was held in sterilised forceps and passed through the flame a few times; with a sterile scalpel the superficial part of carious dentine was removed and the deeper part emulsified in broth pH 7·6. After twenty-four hours' incubation broths of varying degrees of acidity were heavily inoculated, and from these agar plates were inoculated after twenty-four or forty-eight hours' incubation. At first the acid broths used varied from pH 7 to pH 4·5, but as a large variety of organisms grew in each tube higher concentrations of acid up to pH 1 were tried. Bacilli were isolated with some frequency from pH 3 broths, and on one occasion from pH 1, but on the whole we found there was no need to employ such degrees of acidity. Later the carious dentine was emulsified in pH 6 broth direct, and after twenty-four hours' incubation the more acid broths of pH 4·5 to pH 3 were inoculated. The most successful method, however, was to emulsify the carious material in pH 3·5 broth in the first case, as in this the vast majority of tooth organisms fail to grow.

#### *Nature of Micro-organisms Isolated.*

The organisms isolated by the above method fall, morphologically, into two main groups: (a) Type I, a long thin bacillus,  $0\cdot75 \times 2\text{--}3\mu$ , which occurs singly, in pairs or chains, having a marked tendency to parallelism or palisade formation in dried films (Figs. 2 and 3); and (b) Type II, a shorter bacillus,  $0\cdot75 \times 1\text{--}2\mu$ , usually occurring in chains (Fig. 4). After prolonged subculture on agar Type I fails to form chains. Both organisms are non-motile and Gram-positive; both are aërobes and facultative anaërobes (in gelatin stab cultures there is equal growth at the top and the bottom). In broth there is in most cases uniform turbidity, but in a few cases the growth settles to the bottom. On ordinary agar the colonies may appear as minute points after twenty-four hours' incubation, but often do not become visible till after forty-eight hours; they are small, round, greyish and opaque, with a finely granular appearance under the low power and a regular outline (Fig. 5). In size the colonies are about 0·5–1·0 mm. in diameter; on serum agar rather larger, up to 2·0 mm. In a gelatin-agar shake culture the colonies have a rough biconvex appearance which might be described as being "tam-o'-shanter" shaped (Fig. 6).

When first isolated subculturing should be carried out once a fortnight, as the organisms have a tendency to die out, but after prolonged subculture once a month is sufficient.

Type I was isolated in pure culture from the deeper layers of carious dentine from 38 out of 50 teeth examined, and Type II from 18 teeth out of 50. In some cases the growth of mouth organisms in the original broth culture outgrew the above two types to such an extent that they were not isolated although morphologically similar organisms were seen in films; inoculation into acid broth direct obviates this difficulty. Including these cases, Type I "occurred" in 44 cases, *i.e.* 88 per cent., and Type II in 21 cases, *i.e.* 42 per cent. In some cases only one type was isolated from each tooth, in others both types; one or other or both types were isolated in pure culture from 88 per cent. of cases and "occurred" in 96 per cent.

As regards the fermentation of sugars, of the 38 strains of Type I isolated 33 formed acid and no gas in glucose and lactose, no acid nor gas in saccharose

and acid and clot in milk. The milk usually became acid on the second day and was firmly clotted on the third, with all except the top quarter to half an inch completely decolourised. Four of the remaining five strains formed acid in saccharose and glucose, but differed in their reactions in lactose and milk; one strain formed acid in glucose alone. The action of Type II on sugars is nothing like so constant; seven out of the eighteen strains isolated formed acid and no gas in glucose, lactose and saccharose, and acid and clot in milk, the effect on the last-named being similar to that of Type I. Three strains formed no clot in milk after prolonged incubation, and two strains had no effect at all on milk. The remaining six strains varied considerably in their reactions (Table I).

TABLE I.—*Sugar Reactions.*

	Glucose.	Lactose.	Saccharose.	Milk.	Gelatin.	Indol.	No. of strains.
Type I.	Ac. .	Ac. .	0	Ac. Cl. .	0	0	33
	Ac. .	Ac. .	Ac. .	0	0	0	2
	Ac. .	0	Ac. .	Ac. .	0	0	1
	Ac. .	0	Ac. .	0	0	0	1
	Ac. .	0	0	0	0	0	1
Type II.	Ac. .	Ac. .	Ac. .	Ac. Cl. .	0	0	7
	Ac. .	Ac. .	Ac. .	Ac. .	0	0	3
	Ac. .	Ac. .	Ac. .	0	0	0	2
	Ac. .	Ac. .	0	Ac. .	0	0	1
	Ac. .	0	Ac. .	0	0	0	2
	Ac. .	0	0	Ac. Cl. .	0	0	1
	Ac. .	0	0	Ac. .	0	0	1
	Ac. .	0	0	0	0	0	1

In no case was gelatin liquefied by either type, nor was indol formed; twenty-six strains were also inoculated into dextrin and dulcitol without the formation of acid or gas.

We propose to give the name *B. acidophilus odontolyticus* I and II to these bacilli.

Occasionally other organisms were isolated, but with no regularity.

#### *Relation of the Two Types to other Bacteria found in Dental Caries.*

Morphologically Type I very closely resembles one of the organisms illustrated by Miller in an excellent micro-photograph of artificial caries. Howe and Hatch state that the predominating group of organisms in dental caries is the Moro-Tissier group. *B. acidophilus* (Moro) as depicted by them bears morphological resemblance to Type I. The sugar reactions, however, do not agree, since he states that most strains ferment saccharose; the action on milk is not mentioned, but Moro (1905) states that cow's milk is coagulated while human milk is not.

Kligler, working on oral micro-organisms, isolated fifty-eight strains of *B. acidophilus* (Moro), which he states ferments glucose readily with the formation of a considerable quantity of acid, lactose not so readily and



saccharose not constantly. As regards milk, the lower part is coagulated first and litmus milk becomes decolourised. Summarised, the following are the reactions of the strains isolated by him :

No. of strains.	Glucose.	Lactose.	Saccharose.	Milk.	Gelatin.	Indol.
3	+	0	0	0	0	0
35	+	+	0	A.C.	0	0
20	+	+	+	A.C.	0	0

That is, 60 per cent. agree in the sugar reactions with our Type I.

### *The Occurrences of Types I and II in "Normal" Mouths.*

In order to form some idea as to the frequency of occurrence of decalcifying organisms in the mouths of persons who had at one time or another suffered from caries, ten persons thoroughly rinsed their mouths with 10 c.c. of normal saline, and the fluid was collected and deposited into an equal quantity of double-strength broth. From these cultures Type I organisms were isolated in 30 per cent. of cases and Type II in 20 per cent. This experiment will be further described in a later paper, along with the exact relation of the bacilli and their distribution.

### *Agglutination.*

To test agglutination properties, two strains of Type I were inoculated repeatedly into rabbits; each strain agglutinated with its own antiserum up to a dilution of more than 1 in 2560. Sixteen strains were put up against serum A, and 14 against serum B, with the following results :

		No. of strains agglutinating with various serum dilutions.						
	No. put up.	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560
Serum A	16	16	15	12	12	10	8	9
Serum B	14	14	14	13	8	5	5	6

These results include strains of both types, but in each case the organisms which were agglutinated in the higher dilutions belonged to Type I.

### *Final pH Values of Cultures.*

It has been stated above that we found that a concentration of acid greater than pH 4 was necessary to effect decalcification of enamel, therefore it was essential to discover whether the organisms isolated were capable of forming this amount of acid by the fermentation of carbohydrates. Nine strains picked at random were inoculated into glucose broth of pH 7.6 and placed in the incubator until they died out, and the final pH values were estimated on the hydrogen electrode; these varied between pH 3.4 and pH 2.2, the average being pH 2.75. It thus appears that organisms of either Type I or Type II are able to elaborate enough acid from the fermentation of carbohydrates to attack the enamel of teeth. A seven days culture of *B. coli* in glucose broth gave a final pH value of 4.0.

## PRODUCTION OF ARTIFICIAL CARIES.

*Decalcification of Enamel.*

Attempts were next made to produce caries artificially by the action of the organisms isolated. Non-caries teeth were placed in broth and 2 per cent. glucose broth in 3" x 1" tubes and sterilised; various strains were then inoculated into the broth, *S. salivarius* in both ordinary broth and glucose broth and *B. coli* in glucose broth being used as controls. Every eighth day the teeth were removed and placed into fresh uninoculated broth under sterile precautions, so as to approximate somewhat to the conditions in the mouth and prevent the teeth remaining continually in an acid medium. As pointed out by Miller, the first sign of caries is the loss of transparency of the enamel, which becomes opaque; this he attributes to pure decalcification. This opacity is clearly seen in the tooth in Fig. 7, which had been in a broth culture of Type I for seven weeks. Figs. 8 and 9 show low- and high-power magnifications of a longitudinal section from a tooth, the upper half of which had been in a glucose broth culture of Type I for seventeen and a half weeks. The organisms have penetrated the dentinal tubules for a considerable distance *from the pulp cavity side*, which fact proves that the appearance is artificial, since "natural" caries never starts from the pulp cavity and works outwards. In the plates not only can the organisms be seen passing down the tubules, but also a large liquefaction focus can be seen. Fig. 10 is another tooth in which the dentinal tubules have been cut transversely; some of these are seen to be packed with organisms and dilated to a far greater diameter than the empty tubules, which have simply undergone decalcification during the process of embedding. This tooth shows the result of eleven weeks in a glucose broth culture. In all cases observed up to the present the first sign of caries—that is, the opacity of the enamel—occurs on the cutting edge of incisors or canines and on the tip of the cusps of molars and premolars; this fact was also noted by S. P. Mummery (1909–10) when working with teeth suspended in 0·075 per cent. lactic acid. The control teeth in cultures of *S. salivarius* show no macroscopical changes after five months, nor does the control in a culture of *B. coli* after two and a half months; these experiments are being allowed to continue yet further. All the teeth in broth cultures show some slight opacity of the enamel. Further experiments are also being carried out in glucose broth with the lower part of the tooth sealed over in order to produce caries through the enamel alone. Commencing caries of the dentine from the enamel side is indicated in Figs. 12 and 17. Fig. 11 represents a section of a tooth with the tubules cut transversely, showing the effect of placing decalcified non-caries dentine in a broth culture of *S. aureus* for three weeks. No organisms can be seen but the proteolytic enzyme has had a very marked effect, producing enormous cavities in the dentine, far larger than the liquefaction foci which appear in sections of "natural" caries. For the sections and micro-photographs in Figs. 12 to 19 we are indebted to Mr. J. Howard Mummery; these teeth had been in glucose broth cultures for fourteen weeks.



*Penetration and Liquefaction of Decalcified Dentine.*

Although in no case was gelatin liquefied nor indol formed by any of the organisms isolated, Figs. 8, 9 and 16 clearly show the liquefaction foci as described by Miller. After completely blocking the tubules the organisms have produced large cavities in the decalcified dentine in several places. It thus appears that when completely deprived of carbohydrates the organisms isolated can exert a liquefying action on the collagen matrix of the dentine, although as yet we have been unable to obtain any action on gelatin.

Animal experiments are being carried out, the results of which will be discussed in a later paper.

## DISCUSSION OF RESULTS.

In order to facilitate the correlation of our results with the findings in natural caries a short account of the natural process may not be out of place. The main histological features of caries are definite in type and well known.

Dental decay is, with a few exceptions, universally present in both primary and secondary dentition. The condition commences in the crowns of the teeth usually at the points of contact or in the fissures. Caries of the enamel appears as a white opaque area; this is soon followed by fracture of the enamel prisms, disintegration of the enamel and exposure of the dentine. The dentine is then involved, decalcified, softened and destroyed. Microscopically the bacteria can be seen to have penetrated the dentinal tubules, which are enlarged and breaking down with intercommunicating areas. Ground sections of carious teeth at different stages of the disease show the whole process very clearly. There are, however, variations according to the structure of the teeth.

In many of these preparations bacilli morphologically resembling those we have described are seen alone or with other bacteria. Some of Miller's original photographs show this very clearly.

*Enamel changes in artificial caries.*—The primary lesion produced by prolonged contact with cultures of our micro-organisms is a whitish opacity similar to that seen in natural caries. Sections show that this change is not uniform in that in certain areas the penetration is much more marked than in others; this is clearly seen in Figs. 12 and 17—sections of a tooth which had been in a broth culture for seven weeks. The sections also show slight undermining of the enamel. Under the microscope the enamel prisms are granular and in places fragmented.

*Dentine changes in artificial caries.*—Miller noted that the first change was an area of decalcification in advance of the organisms. Fig. 17, a ground section, shows staining of the decalcified dentine, whilst the infected dentine is still more deeply stained. So far we have not been able to get a good specimen showing this passage from the enamel side, but this is mainly due to the fact that we did not leave the teeth long enough in contact with the cultures.

The characteristic mode of spread, however, is well shown in Figs. 8 and 9, and only differs from true caries in that the process started from the pulp surface. Microscopically the bacilli are found to be spreading down the dentinal tubules in a manner identical with the natural process. There is



FIG. 1.

Teeth placed in acid broths of pH 5.0 to pH 1.0 to show the acidity at which enamel is attacked. Those in pH 1, 2, 3, enamel attacked; those in pH 4, 4.5, 5, enamel not attacked.

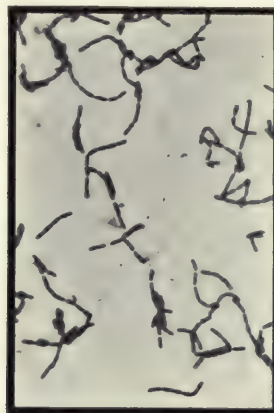


FIG. 2.

Type I bacillus; smear preparation from agar slope culture, showing palisade and chain formation. Gram.  $\times 1000$ .

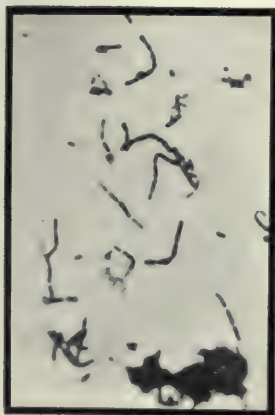


FIG. 3.

Same description as Fig. 2.

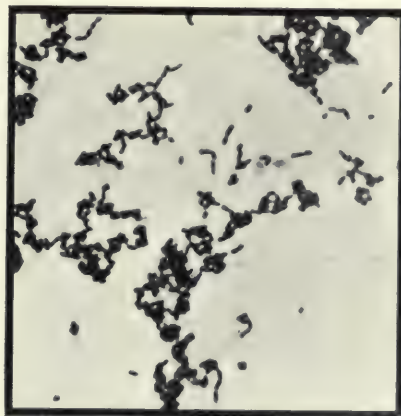


FIG. 4.

Type II bacillus; smear preparation from agar slope culture. Gram.  $\times 1000$ .





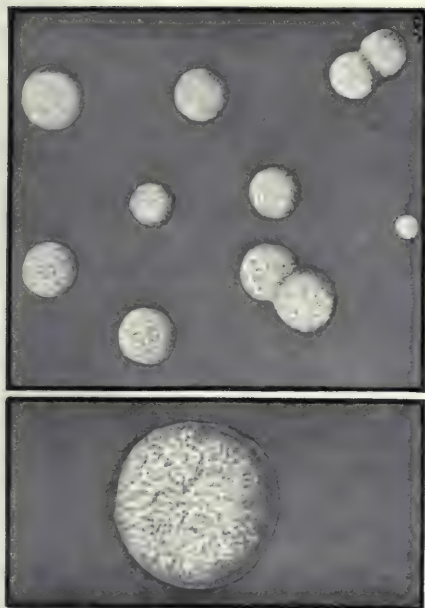


FIG. 5.  
Surface colonies of Type I on agar, showing finely granular appearance.  $\times 18$  and  $\times 45$ .

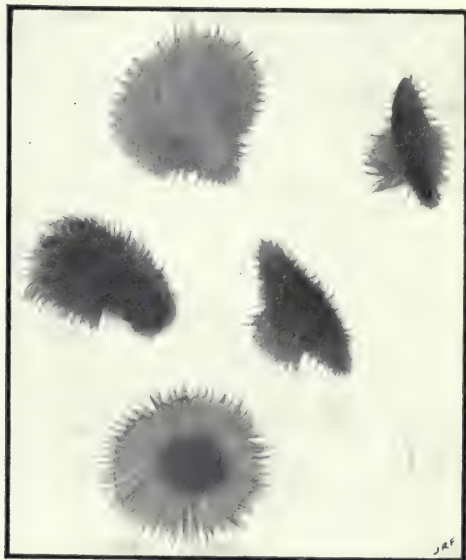


FIG. 6.  
Colonies of Type I in gelatin-agar shake culture; "tam-o'-shanter" colonies.  $\times 45$ .



FIG. 7.  
Artificial caries, showing opacity of enamel. Tooth in broth culture of Type I bacillus for 7 weeks.

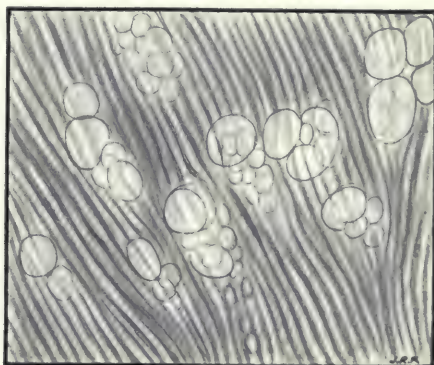


FIG. 11.  
Transverse section of dentinal tubules to show the effect of liquefaction of decalcified dentine by *Staphylococcus aureus*. (Zeiss obj.  $\frac{1}{8}$  in., oc 4.)







FIG. 8.  
Artificial caries; organisms in the dentinal tubules and liquefaction focus. Longitudinal section.  
(Zeiss obj.  $\frac{1}{8}$  in., oc. 4.)



FIG. 9.  
Same specimen as Fig. 8. (Zeiss obj.  $\frac{1}{2}$  in., oc. 4.)

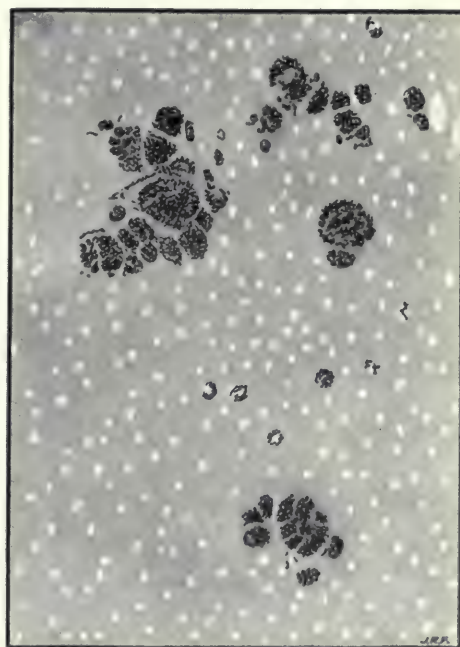


FIG. 10.  
Artificial caries; tubules blocked with organisms and dilated. Transverse section. (Zeiss obj.  $\frac{1}{8}$  in., oc. 4.)





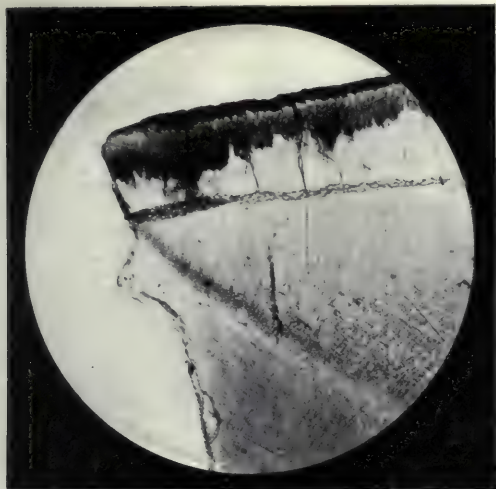


FIG. 12.

Artificial caries; ground section, showing caries of enamel and organisms passing along the amelo-dental junction.  $\times 30$ .

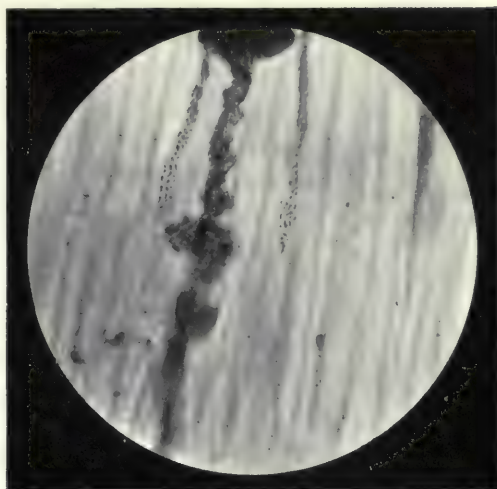


FIG. 13.

Section of artificial caries; individual bacilli in the dentinal tubules, also liquefaction focus.  $\times 700$ .

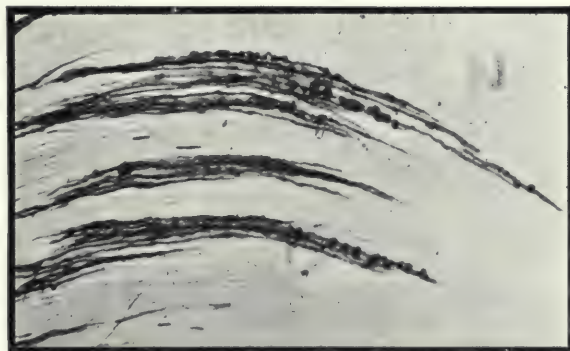


FIG. 15.

Artificial caries; section showing spread of bacilli along the dentinal tubules, which are considerably dilated.  $\times 40$ .

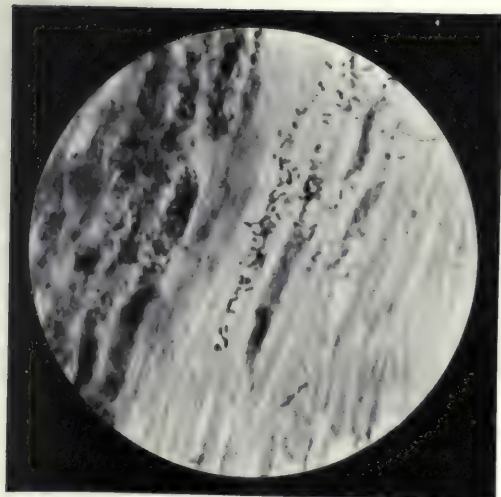


FIG. 14.

Same description as Fig. 13.  $\times 1000$ .

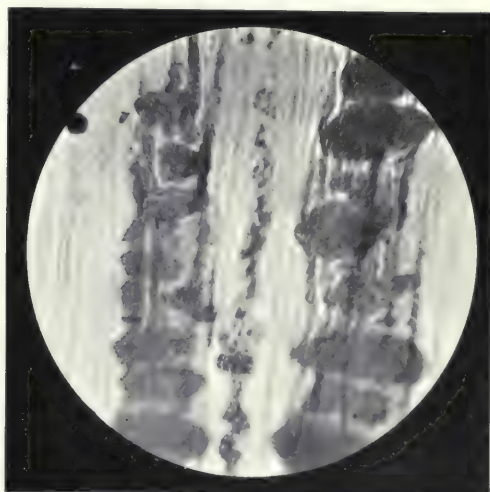


FIG. 16.

Section of artificial caries; large liquefaction foci.  $\times 400$ .





FIG. 19.

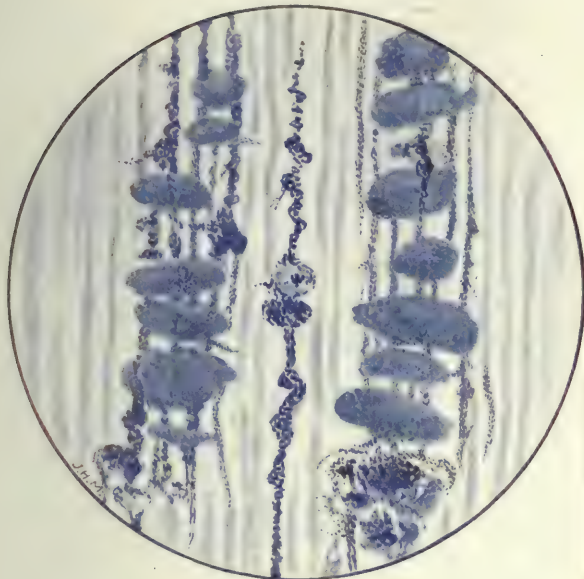


FIG. 19.

Artificial caries; organisms in the tubules and large liquefaction foci. Polychrome methylene blue.  $\times 400$ .

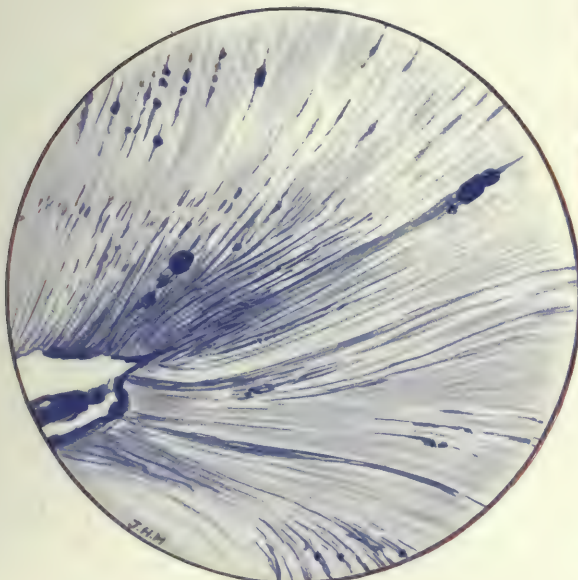


FIG. 18.



FIG. 17.

Ground section showing caries of the enamel and the undermining of the enamel by the organisms, and commencing destruction of the dentine.  $\times 10$ .

FIG. 18.

Artificial caries starting from the pulp cavity; tubules packed with organisms and showing liquefaction foci. Polychrome methylene blue.  $\times 40$ .





also a true widening of the tubules with destruction and formation of liquefaction foci. Those foci stain differently with polychrome methylene-blue from the tubules merely blocked with organisms, the former being greenish in colour and the latter blue (Fig. 19).

The production of liquefaction foci strongly supports the ætiological importance of the bacilli we have described. In addition to producing sufficient acid to decalcify teeth and to initiate dental caries they can liquefy dentine. In natural caries, however, it is probable that this liquefaction process may be aided by a secondary infection of the dentine by proteolytic bacteria, although actively proteolytic bacteria such as the anaërobe *B. sporogenes* were never found. Placing decalcified dentine in cultures of proteolytic bacteria produced an extreme degree of proteolysis with the tubules all widely dilated (Fig. 11), and if the action be prolonged the dentine is completely dissolved. Such a condition is never seen in natural caries.

In a further paper we propose to deal with some of the secondary factors in the production of dental caries.

#### CONCLUSIONS.

(1) The examination of selected carious material showed the constant presence of a definite type of bacillus.

(2) The bacilli are capable of producing a high degree of acidity by the fermentation of carbohydrates. The average final pH value of nine strains was 2.75, which is sufficient to decalcify teeth.

(3) Teeth left in contact with pure cultures over prolonged periods showed changes almost identical with those found in natural caries.

(4) Such teeth show erosion of the enamel with penetration of the dentinal tubules and the formation of liquefaction foci.

(5) The bacilli, to which we propose to give the name *B. acidophilus odontolyticus*, in their resistance to and formation of acid resemble the "acidophilus" group of Moro; biologically, however, there are several points of difference.

Finally we wish to express our indebtedness to Mr. J. Howard Mummery for the great trouble he has taken in the preparation of a number of the sections and drawings, and to Mr. J. Q. Rowett, who has generously defrayed the expenses of this research.

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## ON ISOHÆMAGGLUTINATION.

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THE hypothesis put forward by von Dungern and Hirschfeldt (1911) to explain the facts of isoagglutination between human bloods is now generally accepted. According to this hypothesis, only two agglutinable factors residing in the corpuscles and two agglutinating factors residing in the serum are recognised. According as to the manner in which these factors are distributed, so is the group of any given blood determined. If the agglutinable factors resident in the corpuscles be denominated A and B respectively and the corresponding agglutinating factors resident in the serum a and b, their distribution in the various groups will be as below:

	Serum.	Corpuscles.
Group I . . . . .	<i>Nil</i>	A + B
Group II . . . . .	b	A
Group III . . . . .	a	B
Group IV . . . . .	a + b	<i>Nil</i>

If the above arrangement be the correct explanation of isohæmagglutination, it is obvious that, by saturation with corpuscles, it should be possible to remove the agglutinins corresponding to them from suitable sera. Von Dungern and Hirschfeldt do not appear to have tested their hypothesis in this way. This has been done by Koeckert (1920) with corpuscles and sera of all groups, and by Schütze (1921) for Group IV serum, in both cases with results in agreement with von Dungern and Hirschfeldt's hypothesis. The present paper is in confirmation of these findings.

## TECHNIQUE.

The experiments performed consisted in the absorption of the three agglutinating sera, those of Groups II, III and IV, by the three agglutinable types of corpuscles, those of Groups I, II and III.

The sera used were, for the most part, from a day to a week old, though two of them had been kept in the ice-chest for eighteen months. Some had chloroform added, others were free from preservative; none of these factors appeared to have any substantial bearing upon the results. The corpuscles were collected in 2 per cent. sodium citrate solution in normal saline, washed

three times in normal saline and suspended in the same fluid. At first only fresh corpuscles were used, but it soon became evident that age made little difference to their activity. On standing for some days in normal saline a certain amount of laking took place, but on washing and re-suspending in fresh saline it was found that the corpuscles were as agglutinable and as actively absorbent of agglutinin as when fresh. They appeared to retain their characteristics in this respect until completely destroyed by laking.

The corpuscles were used in a 50 per cent. suspension in normal saline; for absorption five drops of this suspension were added to five drops of the serum to be tested and left standing, either overnight at room temperature, or in the incubator at 37° C. for two hours.

On completion of absorption the mixture of corpuscles and serum was shaken up and the corpuscles then thrown down by centrifuging. The supernatant fluid was pipetted off and tested as to its remaining agglutinating power. For this purpose one drop of the serum was added to one drop of a 1 in 400 suspension of the corpuscles against which it was to be tested, the two thoroughly mixed with a platinum loop and one loopful of the mixture then used to make a hanging drop preparation, which was examined under the lowest power of the microscope. The 1 in 400 dilution of the corpuscles was made by diluting a 50 per cent. suspension to the extent of 1 in 200 in the red cell pipette of a Thoma hæmacytometer. This dilution was used because it was found that with thicker corpuscular suspensions the density of the suspension tended to obscure slight degrees of agglutination. By this method the corpuscles are finally examined in a dilution of about 1 in 800, while the serum is acting in a dilution of 1 in 2; using it, it was found possible to detect agglutinins whose presence was not shown by any other means. The final reading of the hanging drop preparation was made at the end of half an hour, during which time it was occasionally agitated.

The following are examples of the experiments performed :

*Experiment 1.—On Serum II (Agglutinin b).*

Control : Serum II (H) *plus* Corpuscles I (Tr), *plus* Corpuscles III (Co).

Agglutination                    + + +                    + + +

(The letters in brackets denote the individuals from whom the material was obtained.)

The serum was now saturated with Corpuscles I (Tr) (Agglutinable Factors A + B), and Corpuscles III (Co) (Agglutinable Factor B).

After absorption the serum reacted—

	<i>Plus</i> Corpuscles I.	<i>Plus</i> Corpuscles III.
Absorbed by Corpuscles I, Agglutination	0	0
Absorbed by Corpuscles III, Agglutination	0	0

That is to say, both Corpuscles I (A + B) and Corpuscles III (B) remove the Agglutinin b from the serum.

*Experiment 2.—On Serum III (Agglutinin a).*

Control : Serum III (K) *plus* Corpuscles I (Tr), *plus* Corpuscles II (W).

Agglutination                    + + +                    + + +

The serum was now saturated with Corpuscles I (Tr) (Agglutinable Factors A + B) and Corpuscles II (W) (Agglutinable Factor A).



After absorption the serum showed—

	Plus Corpuscles I.	Plus Corpuscles II.
Absorbed by Corpuscles I, Agglutination	0	0
Absorbed by Corpuscles II, Agglutination	0	0

In this case both Corpuscles I (A + B) and Corpuscles II (A) removed the Agglutinin a from the serum.

*Experiment 3.*—On Serum IV (Agglutinins a and b).

Control :

Serum IV (D) *plus* Corps. I (Tr), *plus* Corps. II (Re), *plus* Corps. III (P).

Agglutination      + + +      + + +      + + +

The serum was now saturated with Corpuscles I (Tr) (Agglutinable Factors A + B), Corpuscles II (Re) (Agglutinable Factor A) and Corpuscles III (P) (Agglutinable Factor B).

After absorption the serum showed—

	Plus Corps. I.	Plus Corps. II.	Plus Corps. III.
Absorbed by Corps. I, Agglutination	0	0	0
Absorbed by Corps. II, Agglutination	+	0	+
Absorbed by Corps. III, Agglutination	+	+	0

In this case Corpuscles I (A + B) removed both Agglutinins a and b from the serum ; Corpuscles II (A) removed a, leaving b ; while Corpuscles III (B) removed b, leaving a.

These results are in conformity with von Dungern and Hirschfeldt's hypothesis.

In performing these experiments the sera used were as follows : Of Group IV, 8 ; of Group II, 8 ; and of Group III, 4. Corpuscles were used in various permutations and combinations with the sera from between two and three dozen individuals, of whom two belonged to Group I, four to Group III, and the remainder to Group II.

While making the above investigations, it was found that, though in the majority of instances the degree of saturation of the serum with the corpuscles already described was sufficient to remove all the agglutinins, this was not always the case. In the exceptions, however, the agglutinins were susceptible to being removed by a further saturation with the corpuscles. These facts pointed to differences in either the agglutinating powers of sera of the same groups, or of the agglutinable faculty of corpuscles of the same group, or of both factors.

To throw some light on this question, the relative agglutinating powers of a number of different sera were tried against the same corpuscles and, *vice versa*, the extent to which different corpuscles were agglutinated by the same serum was investigated. The sera and corpuscles used in each individual experiment were of the same age and free from preservatives ; the corpuscles were used in a 1 in 75 dilution of a 50 per cent. suspension, both in normal saline ; the test was performed in small agglutination tubes, the dilutions being made by the drop method. Five drops each of the corpuscular suspension and of the serum in dilutions of 1/2, 1/5, 1/10, 1/20, 1/50 and 1/100 were thoroughly mixed in the tubes, which were then allowed to stand overnight at room temperature ; the readings were taken after thorough agitation by rolling the tubes between the palms. The readings were taken by daylight and with the naked eye only, and all doubtful results were ignored.



Four grades of agglutination were recognised and recorded as follows: Conglomeration of the corpuscles into a solid mass not broken up by agitation + + + ; conglomeration into a few large masses + + ; a suspension of smaller masses in a clear fluid + ; those cases where the fluid could not be said to be clear to the naked eye, but where, nevertheless, macroscopic masses were visible ±.

Of the tests for the relative agglutinating powers of sera from different individuals the following is an example :

		1/2.	1/5.	1/10.	1/20.	1/50.	1/100.
Corpuscles II (W) <i>plus</i>	Serum IV (D)	+++	++	±	—	—	—
	„ (O)	±	—	—	—	—	—
	„ (H)	+++	++	++	±	—	—

These results show clearly that considerable differences exist in the titre of the a agglutinin of Group IV sera from different individuals; similar results showing differences in the titre of the b agglutinin also were obtained. It was, moreover, ascertained that, in a Group IV serum from the same individual, the relative titre of the a and b agglutinins might be very different; in some sera the titre of a and b was found to be about equal, in some a was more powerful than b, and in others the reverse was the case.

Sera belonging to Groups II and III from different individuals were also tested and found to vary greatly in their agglutinin titre. The following is an example from the Group II series :

		1/2.	1/5.	1/10.	1/20.	1/50.	1/100.
Corpuscles III (P) <i>plus</i>	Serum II (W)	+++	++	++	++	+	±
	„ (Re)	+	±	—	—	—	—
	„ (Tr)	++	+	±	—	—	—

As with the sera, so it was found to be the case with the corpuscles, the agglutinability of those of the same group from different individuals was found to be subject to considerable variation, and, moreover, in the case of Group I corpuscles, it was ascertained that the A and B factors might be present in very different degrees. One of the Group I bloods examined gave rise to much trouble before being finally classified, for the reason that the A factor was present to such a small extent as to render the corpuscles inactive to any but a very powerful a agglutinin. This blood was first classified as Group III, and it was only the irregular behaviour of the corpuscles in absorption tests which led to further investigation and its ultimate relegation to Group I.

The following are examples of the results obtained by agglutinating corpuscles from different individuals belonging to the same group, with the same serum :

Serum IV (O) diluted		1/2.	1/5.	1/10.	1/20.	1/50.	1/100.
<i>Plus</i>	Corpuscles II (B)	±	—	—	—	—	—
„	„ (W)	+++	++	±	—	—	—
„	„ (Te)	+++	++	+	±	—	—
Serum II (R) diluted		1/2.	1/5.	1/10.	1/20.	1/50.	1/100.
<i>Plus</i>	Corpuscles I (Da)	+++	+++	++	+	±	—
„	„ (Tr)	+	±	—	—	—	—

Serum III (K) diluted	1/2.	1/5.	1/10.	1/20.	1/50.	1/100.
Plus Corpuscles I (Da)	+++	+++	++	±	—	—
„ „ (Tr)	+++	+++	+++	++	+	±

## CONCLUSIONS.

I. Absorption tests performed on sera of Groups II, III and IV, using corpuscles of Groups I, II and III, confirm von Dungern and Hirschfeldt's hypothesis as to the distribution and nature of agglutinins and agglutinable factors in the four blood groups.

II. (a) Sera from different individuals of the same group vary in their agglutinating powers.

(b) The relative titre of the two agglutinins in any given Group IV serum may be equal or very unequal. Sera from different individuals of this group differ greatly in this respect.

(c) Corpuscles from different individuals of the same group vary as to their agglutinability by the same serum.

(d) The relative degree to which the agglutinable factors may be present in corpuscles from any individual belonging to Group I varies greatly; corpuscles from different individuals belonging to Group I differ in this respect.

The practical importance of the conclusions under II lies in their application to the choice of Groups II and III sera for purposes of blood grouping. For this it is necessary to have sera of a high agglutinin titre, otherwise errors, particularly in the missing of Group I cases, will occur. On this account a quantitative estimation of the agglutinin titre should be made before any serum is selected for grouping purposes, and only those sera should be used which show agglutinating power up to a dilution of at least 1/10.

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## THE RELATIVE VALUE OF HUMAN AND GUINEA-PIG COMPLEMENT IN THE WASSERMANN REACTION.

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It is well known that certain active-serum methods, in which the serum to be tested provides the necessary complement, give a more delicate test in what are known as borderland cases—that is they give a positive result more readily when only a small quantity of syphilitic antibody is present. This greater delicacy is attributed to several factors acting singly or together, namely (1) the absence of the effect of heat in destroying a moiety of the specific antibody, (2) the absence of a similar effect on a non-specific anti-complementary property.

In the course of a duplicate series of tests, using an active serum method in one and the full technique with guinea-pig complement in the other, certain observations suggested another hypothesis, namely that the difference might be due to a different combining value of human complement as compared with that of the guinea-pig, with the complex syphilitic antigen plus antibody. A different combining value of various complements in this respect was noted by Noguchi and Bronfenbrenner (1911)<sup>2</sup>, who found that among several domestic animals rabbit's complement effected a loose combination while that of the goat did not combine at all.

### EXPERIMENT 1.

Experiment 1 was arranged to test the hypothesis that the difference in delicacy of an active-serum method and that of the full technique might be due to a different combining value of the complements employed. For this purpose a triplicate series of tests was performed on several batches of sera as they came in from the hospital and venereal disease clinics.

Series A. Active serum method.

„ B. }	Inactivated	{ Full technique with a foreign human complement.
„ C. }	serum	{ Full technique with guinea-pig complement.

As far as possible Series A was made to correspond, in volumes and concentration of materials used, with Series B and C. This correspondence was made practicable by the fact that the volume of serum tested (inactivated as in the full technique) contains, when active as in the active-serum method,



complement equivalent to about 3 minimum hæmolytic doses (m.h.d.) for the volume of sensitised sheep's red cells used in each of the series of tests. A certain percentage of sera are unsuitable with the active serum method owing to absence of natural complement. Fleming's (1909) technique was employed for Series A, but modified to bring the correspondence to as great a degree as possible. Fleming's reaction, a three-tube method, allows of readings of degrees of positivity by varying the strength of the antigen in the second and third tubes. The highest strength of antigen used in Series A corresponded to the strength used throughout in Series B and C, in which an ordinary three-tube method (with varying doses of complement) was employed, *i. e.* Tube 1 contained inactivated test serum, 3 titrated m.h.d. of complement and saline diluent; tubes 2 and 3 contained inactivated test serum, 3 and 5 m.h.d. of complement respectively and antigen. They were incubated for the usual period and fully sensitised sheep's red cells subsequently added to all the tubes and the readings taken in the usual manner. The only difference between Series B and C was the origin of the complement—in B, man, and in C, the guinea-pig.

Series B and C were put up side by side. Series A was put up, as is necessary when using active serum, within 24 hours of collection and in smaller batches. Upwards of 100 sera in several batches spread out over several months were tested in this manner—a number sufficient to bring out any well-marked difference in the readings of the three methods. It is obvious that any accurate quantitative comparison of Series A with B and C is impossible. In A the complement is not titrated and the result is given in terms of antigen fixed instead of in doses of complement fixed, and considerable experience is necessary in making the readings, especially with weakly positive sera. The readings given by Series A were, however, found to tally in a remarkable manner with those given by Series B; such slight variations as were noted could more readily be accounted for by the other variations of the two methods than by the fact that the serum was inactivated in B. A tabulation of the results is not necessary; for practical purposes they were the same. The difference between the readings of B and C was from the first a very striking one, and the experiment using Series B and C in parallel was continued for several months, upwards of 600 sera being tested. In the first part of these comparisons human complement was derived from one individual only; in the later tests several human complements were included as controls to the original in multiple parallel series, these tests being the first part of Experiment 2, which will be outlined later, Experiments 1 and 2 thus overlapping. Pooled guinea-pig sera were employed, as a general rule from three animals.

The results in terms of positive and negative were as follows:

Number of tests	610
Positive with human complement	208
Positive with guinea-pig complement	171

This balance of 37 in favour of fixation with human complement occurred among the borderland cases, primary, tertiary and treated syphilis. In considering the discordant results the following signs of hæmolysis or its absence may be employed: +, +, + denotes complete hæmolysis in all three

tubes, the first sign indicating the control and the second and third the tubes with antigen and 3 and 5 m.h.d. of complement; +, -, - indicates complete absence of hæmolysis in the antigen tubes or a strong positive;  $\pm$  may be taken to indicate degrees of hæmolysis, and so +, -,  $\pm$  will represent a weak positive and +,  $\pm$ , + a doubtful one. Now the method of testing allows of the detection of a difference of not more than of 5 m.h.d.; such a discrepancy has not infrequently been noted, a +, -, - with human complement giving a +, +, + with guinea-pig complement. As a rule the discrepancy might be accounted for by a different fixing value of from 2 to 4 m.h.d., a reading of +, -, - being converted into +, -, +, or +,  $\pm$ , +, and a reading of +, -,  $\pm$  into +,  $\pm$ , + or +, +, +. In no duplicate series was there a closer approximation. An occasional doubtful positive +,  $\pm$ , + with guinea-pig complement, in which the anti-complementary factor was evidenced by a prolonged delay in the completion of hæmolysis in the control tube, gave a +, +, + with human complement. It may be noted that throughout the experiment the anti-complementary factor to guinea-pig complement was more in evidence than that to human complement. One very exceptional serum from a secondary syphilitic required 12 m.h.d. of human complement to hæmolyse the control tube; 24 m.h.d. of guinea-pig complement was insufficient.

In the experiment so far recorded the evidence in favour of greater fixation with human complement is derived from readings with weekly positive sera. In the following table is shown the result of titrating the strength of a strongly positive serum by dilution of the three essential ingredients.

TABLE I.—*Quantitative Estimates of the Reacting Power of a Syphilitic Serum Using Human and Guinea-pig Complement.*

(a) By dilution of complement. Serum (1 in 5) and antigen (1·5 per cent.) constant.

Control 3	6	7	8	9	10	11	12	13	14	m.h.d. complement.
+	—	—	—	—	$\pm$	$\pm$	$\pm$	$\pm$	+	human.
+	—	$\pm$	$\pm$	$\pm$	$\pm$	+	+	+	+	guinea-pig.

(b) By dilution of antibody serum (preliminary dilution 1 in 20 instead of the usual 1 in 5). Three m.h.d. complement and antigen (1·5 per cent.) constant.

60	50	40	30	20	10	5	c.mm. serum.
—	—	—	—	$\pm$	$\pm$	+	human complement.
—	$\pm$	$\pm$	+	+	+	+	guinea-pig complement.

(c) By dilution of antigen. Three m.h.d. complement and serum (1 in 5) constant.

1·5	·75	·37	·18	·09	antigen per cent. total volume.
—	—	—	$\pm$	+	human complement.
—	$\pm$	+	+	+	guinea-pig complement.

Comparative hæmolytic titre of the undiluted complements. Guinea-pig : human :: 5 : 1 in the preliminary titration.

+ represents complete hæmolysis;  $\pm$  gradations from commencing to almost complete; — absence of hæmolysis.



So far these experiments show that in the technique used the homologous complement is more readily fixed than that of the guinea-pig in the combination with syphilitic antigen and antibody, and that the active serum methods may owe their greater delicacy to this more ready fixation rather than to the absence of inactivation of the serum tested. The delicacy of the reaction is, however, of little importance if it does not permit control of the variation in the capacity for fixation of the complements used. Various workers have pointed out a serious defect in the use of guinea-pig complement, which is sufficient to negative any attempts to control the test except with a most elaborate and exclusive technique. This defect is the absence of any ratio between the hæmolytic titre of a complement and its capacity of being fixed in the combination with syphilitic antigen and antibody. There is a very great difference among various guinea-pigs in this respect. Three authorities as to this point may be quoted:

Browning and Kennaway (1919) say "the complement is an important source of variation, since, as was pointed out by Browning and Mackenzie, there is no constant relationship between its hæmolytic power and its capacity for being bound by the mixture of antigen and antibody." "The same positive serum may require from 5 to 16 doses to produce commencing lysis,"—and, "These variations are quite irregular, and depend on factors which cannot so far be rendered constant."

Noguchi and Bronfenbrenner (1911)<sup>1</sup> put up simultaneously 41 complements from 41 guinea-pigs with a syphilitic serum and estimated the amount of complement removed in hæmolytic units. Their figures are:

0 units . . . . .	1 specimen = 2·4 per cent.
1·5 to 3·5 . . . . .	8 specimens = 19·5    ,,
4 to 5 . . . . .	20 specimens = 48·7    ,,
5·5 to 9·5 . . . . .	12 specimens = 29·2    ,,

There is here an ample range—a range somewhat similar to that observed by Browning if the exceptional 2·4 per cent. specimen is omitted—for the disappearance of many positive reactions.

Griffith and Scott (1920) say "of all the reagents in the Wassermann test complement is the least capable of standardisation, and is certainly the reagent which is most often responsible for the discrepancies on different occasions."

Browning uses ox blood, Noguchi human and Griffith and Scott sheep's blood with their appropriate amboceptors as indicator antigen.

## EXPERIMENT 2.

Experiment 2 consisted of testing 44 human complements from 44 individuals to see if the hæmolytic titre bore any relation to the fixing capacity in the presence of syphilitic antigen and antibody, and to determine whether human complements differed from one another in fixing power in a fashion similar to that observed amongst various guinea-pigs by other workers. The tests were done in several batches spread out over several months, and every batch included Complement No. 1 as a control to an unknown complement both as to hæmolytic titre and what may be called syphilitic titre.



Complement No. 1 has been used for several years, and has never been known to vary in the tests from week to week in its hæmolytic titre or in the readings with the many weak positives used as controls in hundreds of batches of tests. The hæmolytic titre was determined with and without antigen for the full period of incubation in the water bath before the addition of sensitised corpuscles. Subsequently to this, duplicate or multiple parallel series of Wassermann reactions were put up with 3 and 5 m.h.d. of the complements and one or more inactivated weakly positive syphilitic sera giving reactions +, -,  $\pm$ . Of the 43 complements tested against No. 1, 41 resembled it so closely as to show at times only varying degrees of hæmolysis in tube 3, such variations as would be accounted for by small differences inherent in the method of standardisation of complement or perhaps in differences of an anti-complementary factor in the active complement-containing serum—a variation which could be expressed in a fraction of a m.h.d. Two complements only, one from a laboratory assistant with phthisis (the result with this one was repeated after an interval of one year), and another from a patient with an extensive boil on the neck, converted a +, -,  $\pm$  to +,  $\pm$ , +, neither of them varying sufficiently to convert a weak positive into a complete negative—a variation in fixing power of 2 m.h.d. Whether or not the illness of these two accounted for a deficient fixing power is not known. The other complements were derived from apparently healthy individuals of ages from 60 to 3 years, mostly males.

The results of the comparative tests showed that for practical purposes with all the healthy human complements used there is a constant ratio between the hæmolytic titre and the syphilitic titre in the Wassermann reaction, and that the fixing power of various human complements does not vary in a manner similar to that observed amongst guinea-pigs.

A few points with regard to the hæmolytic titre of the human complements may be mentioned here. Complement No. 1 (the standard) in the technique employed contains 3 m.h.d. in 10 c.mm.; two human sera contained 2 m.h.d. in 10 c.mm., four contained 4 m.h.d. in the same quantity; the others were identical with Complement No. 1 within the limits of the method of standardisation. The variations in quantities of natural amboceptor for sheep's cells has not been taken into account; this would appear from these and other experiments, not included in this paper, to be of not much account—at any rate with this technique. The human complements were all titrated and used on the day of collection. The tests of the titration with and without antigen of all the complementary sera were most satisfactory; several were tested both without and with the full incubation in the water bath and showed that very little available complement was used up during the incubation. This incubation is of course necessary in view of the possibility of a serum being syphilitic. They all showed that a clear 2 m.h.d. of complement was available for the final tests. It has never been necessary when doing tests with human complement to cease work and look for fresh material, and the completion of the task in hand within the hours allotted to it is always assured, and with the knowledge that a result obtained with the controls of the previous batch of tests will be repeated with certainty with subsequent batches.

It is not proposed to detail in this paper the technique employed. In

relative volumes of materials used and relative periods of incubation it does not differ from other methods. Some essential details are :

- (1) The diluent salt solution.
- (2) Quantities of materials.
- (3) Antigen.

(1) *The diluent salt solution.*—Too much emphasis cannot be laid on this apparently trifling detail. In the early days of working with active-serum methods it was noticed on several occasions that there was an absence of hæmolysis with all or an unusually large percentage of the human sera to be tested. This was traced to absorption of complement by the distilled water used in making up the salt solution. On using freshly distilled water of which the first fraction had been discarded this trouble disappeared, and all the sera were found to give satisfactory hæmolysis. Human complement is of low titre, and it is essential that none of it should disappear in combination with the diluents used in the reaction. The salt solution used in these tests has been made up in a two-litre flask of alkali-free glass with freshly distilled water and the whole thoroughly dissolved and shaken and then autoclaved at 110°C. The flask, being fitted with a hooded pipette and tap, serves for several batches of tests and remains sterile with due care.

(2) *Quantities of materials used.*—Total quantity in each tube = 0·2 c.c., comprising—

Diluted complement 50 c.mm. = 10 c.mm. to 16·6 c.mm. serum.

Diluted test serum 50 c.mm. = 10 c.mm. serum.

Antigen or salt solution 50 c.mm.

3 per cent. sensitised sheep's red cells 50 c.mm. (sensitised with 5 m.h.d. amoceptor).

The amount of human blood necessary to provide complement for titration and the test of 25 to 30 inactivated sera is 3 c.c. The technique in manipulating these small quantities is that of Wright (1921).

(3) *The antigen.*—Both ethyl alcohol and cholesterol are very anti-complementary in the reaction with human complement, and an antigen devised by Fleming and used for the last seven years in this laboratory has been used in these tests. It is free from cholesterol, and its high concentration in the necessary constituents of human heart muscle made possible by the removal of the watery moiety allows of a greater dilution of the alcoholic solvent. It is very simply made up. 100 grm. human heart free from fat is minced and put into a flask with 500 c.c. acetone. After 24 hours at room temperature the solid portion is filtered off and dried in the incubator. This is then put into another 500 c.c. acetone and treated a second time in the same manner. The dried material is then extracted for a week on the bench in 100 c.c. absolute alcohol, and the clear amber-coloured fluid is filtered off and stored for use. Alternatively the dried material may be stored for future extraction and keeps well. A 10 per cent. dilution in saline of this extract should be "crystal clear." Should a marked turbidity result on dilution, as has happened with some samples, the material should be discarded: turbid extracts are more anti-complementary than is desirable. The antigen volume used in the test is as a rule a 7 per cent. dilution—or 1·75 per cent. of the total volume.



## CONCLUSIONS.

(1) Among healthy human complements there is for practical purposes a constant ratio between the hæmolytic titre and the capacity for being fixed with syphilitic antigen and antibody. This constant ratio enables the reaction to be standardised, and in this respect human complement is superior to that of the guinea-pig in the Wassermann reaction.

(2) The fixation of human complement in the reaction is much more complete than that of the guinea-pig and allows of a more delicate test without the use of cholesterinised antigen.

(3) The difference in delicacy observed in the active-serum methods is probably due to this greater fixation of human complement, and not to the absence of inactivation of the serum to be tested.

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## FURTHER EXPERIMENTAL STUDIES ON IMMUNISATION AGAINST *B. DYSENTERIÆ* (SHIGA) AND ITS TOXINS.

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IN a previous communication (Kanai, 1921) experimental evidence has been brought forward to show that rabbits immunised by the subcutaneous administration of a carbolised vaccine of *B. dysenteriae* (Shiga) acquired a more solid immunity to living Shiga bacilli intravenously administered than those immunised *per os*. In the course of this work it was noted that those animals which succumbed to the test inoculation invariably showed some paralysis prior to death, with the constant post-mortem finding of hæmorrhages in the medulla and spinal cord, whereas any pathological change in the intestinal mucosa apart from œdema was of rare occurrence. This mode of reaction on the part of the rabbit to dysentery toxin is well known, and the difference in behaviour of this animal and the guinea-pig to the toxin of *B. dysenteriae* has led to the conclusion that there exist in reality two toxins. The one, especially toxic for the rabbit, gives rise to the paralytic symptoms, the other produces pyrexia and general marasmus and is more readily demonstrated in the guinea-pig. These two toxins have been variously referred to as "rabbit toxin" and "guinea-pig toxin," or "paretische Gift" and "marantische Gift" (Pfeiffer, 1908), and attempts to establish the nature of these toxins have been productive of much discussion. It would be impossible in the space of a short article to review the literature on this question, but suffice it to say that up till quite recently it remained undecided. The recent researches of Olitsky and Kligler (1920), however, would appear to offer a solution of this question in so far as the effect on the rabbit is concerned. These workers claim to have demonstrated unequivocally the presence of two types of dysentery toxin. One is soluble, a true exotoxin, is destroyed by heating at 80° C., and is responsible for the paralytic symptoms in the rabbit. It is essentially a neurotoxin and gives rise to those hæmorrhages in the medulla and cord to which reference has already been made. The other type they classify with the so-called endotoxins. It is more heat-stable than the neurotoxins, withstands a temperature of 80° C., and produces the hæmorrhages in the intestinal mucosa, which determine the characteristic lesion of dysentery.

Besredka (1919) claims, in his recent publication on immunisation by the oral route, that the immunity produced in animals by this method to *B. dysenteriae* (Shiga) is a local immunity. The cells of the intestinal mucosa have acquired a new character; they are immune to dysentery toxin. Was it

possible, then, that those rabbits which in our last study had been immunised *per os* and which were unable to withstand the test-dose of living bacilli, succumbed to the neurotoxin (exotoxin) alone, and that they were immune to the so-called endotoxin, which, according to Olitsky, produces the hæmorrhagic lesions in the intestinal wall? It may be recalled that these animals showed lesions in the central nervous system only. It was decided to test the correctness of this hypothesis.

#### OUTLINE OF THE PROPOSED EXPERIMENTS.

It is well known to all who have studied the toxicity of dysentery bacilli that strains of the same organism differ markedly in their power to produce intestinal lesions in the rabbit. It was proposed first of all, therefore, to see if a strain of *B. dysenteriae* (Shiga) could be found which produced chiefly intestinal lesions. It was thought that with such a strain it would be possible to test the local immunity produced by the oral administration of the vaccine.

TABLE I.

Rabbit.	No. of strain.	Symptoms.	Dysentery bacilli recovered from—	Post-mortem findings.
No. 60. 1400 gr.	151.	Paralysed on 1st day, died 28 hours after injection.	Gall - bladder, spleen and kidney.	<i>Lung</i> : Hæmorrhages. <i>Small intestine</i> : Injection of mucous membrane and leucocytic infiltrations of tunica propria.
No. 61. 1870 gr.	152.	Found dead 19 hours after injection.	Spleen.	No hæmorrhages in lung, otherwise changes similar to those in No. 60 were observed.
No. 62. 1630 gr.	208.	Paralysed on 3rd day. Died on the 5th day. No diarrhoea.	—	<i>Small intestine</i> : Hyperæmia and œdema. <i>Appendix</i> shows similar changes. <i>Large intestine</i> : appears normal.
No. 63. 1580 gr.	753.	Paralysed on 1st day, died on the 2nd day, no diarrhoea.	Gall-bladder, appendix and lung.	<i>Lung</i> : Hæmorrhages. <i>Liver</i> : Congested. <i>Small intestine and duodenum</i> : Some œdema and hyperæmia.
No. 64. 1330 gr.	752.	Found dead next morning.	Gall - bladder, spleen and kidney.	Same as in Rabbit 63.
No. 65. 1840 gr.	754.	Paralysed on 2nd day, died on 3rd day.	Gall-bladder and spleen.	No hæmorrhages in lung or noteworthy changes in gut-wall.
No. 78. 1500 gr.	Besredkas' strain, $\frac{1}{2}$ of an agar slope.	No paralysis and no diarrhoea. Rabbit found dead on the 2nd day.	Gall-bladder and spleen.	<i>Lung</i> : Small hæmorrhages. <i>Liver and kidney</i> : Congested. <i>Small intestine</i> : (Edema and injection of submucosa. <i>Large intestine</i> : (Edema and injection with some hæmorrhages in submucosa and tunica propria.
No. 77. 1380 gr.	The above strain, $\frac{1}{5}$ of an agar slope (weak growth).	No symptoms; survived.		



Further, the preparation of dysentery exo- and endotoxin, on the lines of Olitsky's work, was envisaged, with the object of testing the relative immunity of rabbits, immunised subcutaneously and orally, to these two toxins.

#### COMPARISON OF THE TOXICITY OF VARIOUS STRAINS OF *B. DYSENTERIÆ* (SHIGA).

Seven strains of *B. dysenteriae* (Shiga) were taken and injected intravenously into rabbits in a dose of one-fifth of a 24-hour agar slope culture. This quantity of bacilli was suspended in 1·0 c.c. of normal saline. Those rabbits which died as the result of the inoculation of the living bacilli were examined for the presence of hæmorrhagic lesions—particularly in the intestine—while the gall-bladder, heart-blood, spleen, kidney, lung and liver were submitted to cultural investigation for the presence of dysentery bacilli. The results of this experiment are given in Table I.

Of the seven strains employed in this experiment Besredka's alone appears capable of producing hæmorrhages in the intestine, but even with this strain they were of a trivial character. It was decided, therefore, to proceed with the preparation of exo- and endotoxin with which to test the local immunity of the immunised rabbits.

#### PREPARATION AND CHARACTERS OF DYSENTERY TOXINS.

##### *Exotoxin.*

The growths from fifteen 24-hour agar slopes (strain No. 151) were suspended in 10·0 c.c. normal saline and immediately filtered through a Berkefeld filter-candle (N). The filtrate was tested on rabbits by intravenous inoculation and the results obtained are recorded below (Table II).

TABLE II.

Rabbit.	Body-weight.	Dose injected.	Symptoms.	Post-mortem findings.
No. 88.	1400 grs.	5·0 c.c.	Paralysis of fore-legs 1st day after inoculation. No diarrhoea. Found dead on 2nd day.	<i>Lungs, liver and kidneys</i> are congested. <i>Spleen</i> congested, Malpighian bodies swollen, and show some small hæmorrhages. <i>Large intestine</i> : Edema and injection of submucosa and tunica propria, with some extravasation of red cells in the oedematous portions. <i>Small intestine</i> : Injection and oedema of submucosa. <i>Medulla and spinal cord</i> . Pia oedematous and vessels dilated. Some hæmorrhages in the grey substance and chromatolysis of nerve-cells.
No. 76.	2330 grs.	3·0 c.c.	Dyspnœa 2 hours after injection lasting 2 hours. Hind legs paralysed next morning. Died 2·8 hours after inoculation.	Similar to those observed in No. 88.
No. 77.	2230 grs.	1·0 c.c.	Hind legs paralysed on 2nd day. Died 3 days after injection.	—

It will be seen from this experiment that when a 24-hour agar culture of *B. dysenteriae* (Shiga) is suspended in normal saline and immediately freed from the bacillary bodies by filtration, the filtrate is highly toxic for rabbits, producing the same symptoms and post-mortem changes as the whole bacilli.

*Resistance of "exo-toxin" to heat.*—Exotoxin, prepared as in the previous experiment, was submitted to temperatures of 60° C. and 80° C. for one hour and inoculated intravenously into rabbits in doses of 1·0 c.c., with the following results :

TABLE III.

Rabbit.	Body-weight.	Toxin.	Results.
No. 83.	1320 grs.	1·0 c.c. "exotoxin" heated at 80° C.	No symptoms. Survived.
No. 84	1100 grs.	1·0 c.c. "exotoxin" heated at 60° C.	Paralysis 1st day. Found dead on morning of 2nd day.

Post-mortem findings in Rabbit No. 84:—*Lungs*: Congestion and hæmorrhages. *Spleen and kidney*: Congested. *Liver*: Congested—shows evidence of infection with coccidia. *Small intestine*: Injected. Polynuclear infiltration of submucosa, with some hæmorrhages. *Large intestine, appendix*: Normal except for presence of coccidia.

It is seen, then, from this experiment that the readily soluble portion of the dysentery toxins (exotoxin) is rendered non-toxic for the rabbit by heating it at 80° C. for one hour, whereas a temperature of 60° C. leaves it capable of producing both lesions in the central nervous system and hæmorrhage in the intestinal mucosa.

*Minimum lethal dose of "exotoxin."*—An attempt to determine the M.L.D. of this exotoxin gave inconclusive results. Three rabbits were inoculated intravenously with 0·1 c.c., 0·5 c.c. and 1 c.c. respectively. The two which received 0·1 c.c. and 1·0 c.c. survived, having shown no symptoms whatever, whereas the third with 0·5 c.c. became paralysed on the first day, recovering on the fourth day after inoculation.

#### *Endotoxin.*

It was held by Klein (1907) and Heller (1909) that the toxin of *B. dysenteriae* (Shiga) was of the nature of an endotoxin, but, as already stated, the experiments of Olitsky and Kligler would appear to demonstrate the existence of two dysentery toxins, the one soluble, destroyed at 80° C.—neurotoxin—the other remaining attached to the bacillary bodies, resisting a temperature of 80° C. and giving rise to the hæmorrhagic lesions in the intestine. The following experiments were undertaken to determine the toxicity of dysentery bacilli after removal of the soluble toxin by washing in normal saline. The strains employed were No. 151 and Besredka's.

*Toxicity of bacilli washed in saline five times.*—The growth obtained from fifteen 24-hour agar slopes was suspended in 5·0 c.c. normal saline and



centrifuged to throw down the bacilli. The clear supernatant fluid was pipetted off and an additional 5.0 c.c. saline added, in which the deposit was re-suspended. This was repeated five times. The final suspension was heated at 60° C. for one hour, centrifuged again, and the toxicity of the supernatant fluid and the bacillary deposit tested on rabbits (Table IV).

TABLE IV.

Rabbit.	Body-weight.	Inoculum.	Result.
No. 92.	1500 grs.	500 × 10 <sup>6</sup> washed bacilli.	Survived.
No. 90.	1520 grs.	1000 × 10 <sup>6</sup> washed bacilli.	Hind legs paralysed 2nd day. Killed 3rd day for examination.
No. 91.	1300 grs.	2000 × 10 <sup>6</sup> washed bacilli.	Forelegs paralysed 1st day. Died 2nd day.
No. 93.	1550 grs.	3.0 c.c. supernatant.	Died 2nd day.

Post-mortem findings in rabbit No. 90:—*Lungs*: Congested. *Spleen*: Hæmorrhages in pulp, and some necrosis of the cells in the germ centres. *Liver*: Congested. *Small intestine*: Vessels of submucosa dilated and some œdema of the tunica propria. *Large intestine*: Œdema. Some small hæmorrhages in tunica propria. *Spinal cord*: Numerous small hæmorrhages in grey matter and chromatolysis of nerve-cells.

Rabbit No. 93 showed very similar changes.

*Effect of a temperature of 80° C. on endotoxin.*—The same bacterial emulsion, washed five times (as used in the last experiment), was heated at 80° C. for one hour and three rabbits inoculated intravenously with varying doses. The result of this experiment is given in Table V.

TABLE V.

Rabbit.	Body-weight.	Dose inoculated.	Result.
No. 94.	1210 grs.	2000 × 10 <sup>6</sup>	Survived. No symptoms.
No. 95.	1300 grs.	1000 × 10 <sup>6</sup>	" "
No. 96.	1880 grs.	4000 × 10 <sup>6</sup>	" "

Rabbit No. 96 was killed and examined, the post-mortem findings being as follows:—*Lungs*: Congested. *Spleen*: Pulp congested and shows hæmorrhages. *Kidney*: Hæmorrhages in cortex. *Small intestine*: Injection of vessels of villi and œdema of tunica propria. *Large intestine*: Injection of vessels of tunica propria. Some small hæmorrhages in wall of appendix. *Spinal cord and medulla*: No change.

This experiment was repeated, but a suspension of unwashed bacilli was employed in place of bacilli washed five times in normal saline. The suspension was heated for one hour at 80° C. as before.

TABLE VI.

Rabbit.	Body-weight.	Dose inoculated.	Result.
No. 132.	1920 grs.	5000 $\times$ 10 <sup>6</sup> .	No symptoms.
No. 131.	1900 grs.	10,000 $\times$ 10 <sup>6</sup> .	"
No. 135.	2010 grs.	20,000 $\times$ 10 <sup>6</sup> .	Diarrhœa. No paralysis. Found dead 1st day after inoculation.
No. 136.	2040 grs.	40,000 $\times$ 10 <sup>6</sup> .	Weak 1st day after inoculation. Recovered.

Post-mortem findings in Rabbit 135:—*Lungs*: Hæmorrhages in right lobe. *Spleen*: Severe congestion of pulp and small hæmorrhages in folliculi. *Liver*: Hæmorrhages. Necrosis of parenchyma with leucocytic infiltration of necrotic areas. *Kidneys*: Congestive swelling of convoluted tubules. *Small intestine*: Hyperæmia of tips of villi. *Stomach*: Hæmorrhages in submucosa. *Large intestine*: Œdema and hæmorrhages in tunica propria (heavily infected with coccidia). *Spinal cord*: Small capillary hæmorrhages in grey matter of cervical portion and in medulla.

From the above experiments it would appear that the readily soluble portion of the toxin of dysentery bacilli, the so-called exotoxin, produces changes chiefly in the central nervous system. These are of the nature of capillary hæmorrhages in the grey matter of the cord and medulla and chromatolysis of the nerve-cells. The action of this exotoxin is not confined to the nervous system, however, as a general action on the circulatory system is evinced by capillary hæmorrhages throughout the body. Thus hæmorrhages were noted in the lungs, liver, kidney, spleen, and especially in the submucosa of intestine, which invariably showed some œdema accompanied as a rule by extravasation of red cells. Heating for one hour at 60° C. did not reduce the toxicity of this soluble portion of the dysentery toxin.

From the experiments carried out with the washed bacilli it is evident that a large proportion of the toxin still remains closely united with the bacterial bodies, even after they have been washed five times and extracted with normal saline for one hour at a temperature of 60° C. Bacilli so treated were still highly toxic for rabbits, producing much the same changes as those arising from the inoculation of the soluble portion of the dysentery toxin. When these washed and extracted bacilli are submitted to a temperature of 80° C. for one hour their toxicity for the rabbit is greatly reduced. Of the three rabbits inoculated with bacilli so treated none died or showed any symptoms whatever. The findings in one of these three rabbits, which was killed and examined, showed complete absence of change in the central nervous system, though there were hæmorrhages in the various viscera. These experiments were carried out with strain No. 151, and it must be admitted that the attempts to prepare an exo- and endotoxin from it were not attended with any great success, though bacilli when heated at 80° C. seemed to have lost to a certain extent their neurotoxin and were yet capable of giving rise to hæmorrhages, particularly in the intestinal submucosa. The following experiment was therefore carried out with Besredka's strain, to see whether it would be possible to achieve with another strain of *B. dysenteriæ* (Shiga) what had



proved unsuccessful with strain 151, viz. the separation of exotoxin from endotoxin. This experiment is summarised in Table VII.

TABLE VII.

Rabbit.	Material inoculated.	Result.
No. 123.	Fifth washing of the growth from ten agar slopes. (Bacilli washed each time with 5.0 c.c. saline, fifth washing filtered through a Berkefeld filter N.)	Paralysis of forelegs on 1st day. Died 2 days after inoculation.
No. 124.	The washed bacilli from above, suspended in saline, heated at 60°C. for one hour and centrifuged. Bacilli so obtained inoculated intravenously in a dose of $2000 \times 10^6$ .	Paralysed and died the 1st day after inoculation.
No. 126.	The tenth washing of five agar slope cultures. (Bacilli washed each time with 5.0 c.c. saline, the tenth washing filtered through a Berkefeld filter N.)	Survived. No symptoms.
No. 127.	The bacilli after tenth washing, heated for one hour at 60°C., washed three times with normal saline (5.0 c.c. saline on each occasion). Bacilli so treated inoculated intravenously in a dose of $3000 \times 10^6$ .	No paralysis. No diarrhoea. Died suddenly on 3rd day.
No. 125.	Bacilli as prepared for inoculation of Rabbit No. 127 heated for one hour at 80°C. and inoculated intravenously in a dose of $4000 \times 10^6$ .	Survived. No symptoms.
No. 128.	Twenty-four hour growth on agar suspended in normal saline, heated for one hour at 80°C. and inoculated intravenously in a dose of $10,000 \times 10^6$ .	No diarrhoea. No paralysis. Died 1st day after inoculation.

The post-mortem examination of those rabbits which succumbed revealed precisely the same changes as those produced by similar preparations made with strain No. 151.

It will be seen, then, from the above experiment that the attempt to separate endo- and exotoxin from Besredka's strain of *B. dysenteriae* (Shiga) had not been attended with any more success than in the case of strain No. 151. The experiments, however, demonstrate the fact that when a 24-hour culture of *B. dysenteriae* (Shiga) is suspended in normal saline and the bacilli then removed by centrifugalisation and filtration, the filtrate is highly toxic for the rabbit, giving rise to similar changes to those produced by the whole bacilli. Both these two fractions, the supernatant fluid and the washed bacilli, appear to contain the two toxins, the neurotoxin and the hæmorrhagic toxin, but by heating the washed bacilli for one hour at 80°C. their power of producing lesions in the central nervous system is destroyed. They are still capable, however, of giving rise to hæmorrhages in the various viscera when inoculated intravenously, these changes occurring constantly in the submucosa of the intestine. A temperature of 60°C. is without effect on the toxicity of these fractions of dysentery toxin.

#### RESISTANCE OF IMMUNISED RABBITS TO TOXIN FRACTIONS.

It now remained to test the immunity of rabbits, immunised orally and subcutaneously, to these two fractions of dysentery toxin. Before doing this,

however, it was decided to see if any pathological changes occurred in rabbits as the result of the administration of large doses of dysentery vaccine *per os*. Five rabbits were taken and to each was given one-fifth of a Roux-bottle culture of *B. dysenteriae* (Shiga), killed by heating for one hour at 60° C. These rabbits were killed at varying intervals after the ingestion of vaccine and the post-mortem finding are given below (Table VIII).

TABLE VIII.

Rabbit.	Time of killing.	Post-mortem findings.
No. 97 (1325 grs.).	4 hrs. after ingestion.	Some congestion of appendix, otherwise normal.
No. 99 (1750 grs.).	24 hrs. „ „	Congestion of appendix, and tips of villi in small intestine. Large intestine showed presence of coccidia.
No. 100 (1850 grs.).	48 hrs. „ „	<i>Spleen</i> enlarged, congested. Shows some nodular lesions. <i>Liver</i> shows nodular lesions. <i>Kidney</i> hæmorrhages.
No. 101 (1660 grs.).	96 hrs. „ „	<i>Small intestine</i> : Congestion of submucosa with some petechial hæmorrhages. <i>Appendix</i> : Congestion and eosinophile infiltration—coccidia. <i>Kidney</i> : Congestion. <i>Liver</i> : Hæmorrhages. <i>Spinal cord</i> : Capillary hæmorrhages in cervical portion.
No. 98 (1550 grs.).	2 weeks „ „	<i>Small intestine</i> : Congestion. <i>Large intestine</i> : One or two hæmorrhages. Coccidia. <i>Appendix</i> : Congestion and œdema of tunica propria. <i>Kidneys</i> : Hæmorrhages, with accompanying degenerative changes in parenchyma. <i>Spinal cord and medulla</i> : Congestion and capillary hæmorrhage.

Unfortunately, four out of the five rabbits were infected with coccidiosis, which detracted to a great extent from the value of this control experiment, as attention has frequently been drawn to the production of hæmorrhages in rabbits by this parasite. However, the experiment is not without interest. Undoubtedly some of the hæmorrhages were due to coccidiosis, but in rabbits Nos. 101 and 98 these occurred in kidney and central nervous system, where no parasites could be found. It seems legitimate to conclude, therefore, that these latter hæmorrhages were the result of the ingestion of dysentery vaccine, the dysentery toxin having passed through the intestinal wall into the general circulation.

Six rabbits were immunised by the oral administration of a Shiga vaccine (strain No. 151, killed at 60° C.), the dose employed being one-fifth of a Roux-bottle culture, and three doses being given at 10 days' interval. Another series of six rabbits received 3 doses of a carbolised vaccine of the same strain inoculated subcutaneously, the interval between the injections being again 10 days. The doses employed were: 1st dose,  $50 \times 10^6$ ; 2nd dose,  $100 \times 10^6$ ; 3rd dose,  $200 \times 10^6$ .

Ten days after the last immunising dose four rabbits in each series were given varying doses of the soluble dysentery toxin intravenously, whilst the remaining two animals in each series received intravenous doses of washed dysentery bacilli which had been submitted to a temperature of 80° C.



The dose of washed bacilli (heated to 80° C.) was fixed at  $20,000 \times 10^6$  (from result of experiment detailed in Table VI), but as this proved insufficient to kill the control, Table IX (annexed) gives no information as to immunity to toxic substances present in the washed and heated bacilli.

TABLE IX.

## A.—Rabbits Immunised by Subcutaneous Route.

Rabbit.	Weight.	Test-dose.	Result.
No. 102.	1350 grs.	0.7 c.c. "soluble" toxin intravenously.	Paralysed on 2nd day. Recovered on 4th day. No further symptoms. Killed and examined. <i>Lung</i> : Congestion and hæmorrhages. <i>Spleen</i> : Severe congestion and hæmorrhages in pulp. <i>Liver</i> : Small hæmorrhages. <i>Small intestine</i> : Congestion and œdema of submucosa. <i>Spinal cord and medulla</i> : Hæmorrhages in grey substance.
No. 193.	1650 grs.	0.7 c.c. "soluble" toxin intravenously.	Paralysed on 2nd day. Died on 4th day. <i>Lung</i> : Congestion and serous exudate in alveoli. <i>Spleen</i> : Malpighian bodies show hæmorrhages and œdema of germ centres. <i>Small intestine</i> : Injection of submucosa. <i>Large intestine</i> : Injection of submucosa. <i>Appendix</i> : Eosinophile infiltrate. Coccidiosis. <i>Spinal cord</i> : Vessels of grey substance injected—some hæmorrhages. Nerve-cells show chromatolysis and some vacuolisation. <i>Medulla</i> : Injection of vessels and hæmorrhages in grey substance.
No. 104.	1150 grs.	1.4 c.c. "soluble" toxin intravenously.	Hind legs became paralysed 20 hours after inoculation. Died soon after. <i>Lung</i> : Severe congestion and hæmorrhage. <i>Spleen</i> : Congestion and hæmorrhage. <i>Liver</i> : Congestion and hæmorrhage, some fibrosis and infiltration with eosinophile leucocytes. <i>Small intestine</i> : Submucosa shows injection of vessels, with small hæmorrhages. <i>Large intestine</i> : Tunica propria shows injection of vessels, œdema, eosinophilic infiltrate and many small hæmorrhages. <i>Spinal cord, medulla</i> : Hæmorrhages and degenerative changes in nerve-cells.
No. 105.	1600 grs.	Ditto.	No symptoms. Survived. Killed and examined. <i>Lung, liver and spleen</i> : Congestion. <i>Small intestine</i> : Œdema. Injection of vessels and hæmorrhages in tunica propria. <i>Large intestine</i> : Injection and œdema of submucosa. <i>Spinal cord and medulla</i> : Nil. Rabbit infected with coccidia.
No. 106.	2150 grs.	$20,000 \times 10^6$ washed bacilli killed by heating at 80° C. for one hour.	No symptoms. Survived. Killed and examined. <i>Lungs</i> : Nil. <i>Spleen</i> : Enlarged. Swelling of cells in germ centres. <i>Small intestine</i> : Small macroscopic hæmorrhages. <i>Large intestine</i> : Many small hæmorrhages and œdema of tunica propria. <i>Spinal cord</i> : Injection of vessels in grey matter. <i>Medulla</i> : Nil.
No. 107.	2000 grs.	Ditto.	No diarrhœa. No paralysis observed. Found dead on morning of 3rd day. <i>Lung</i> : Œdema and fibrinous exudate in alveoli. <i>Liver and kidney</i> : Congested. <i>Small intestine</i> : Injection of submucosa. <i>Large intestine</i> : Nil. <i>Spinal cord</i> : Large hæmorrhages in grey matter of cervical and lumbar regions. Nerve-cells show degenerative change. <i>Medulla</i> : Injection of grey matter.

TABLE IX—*continued.*B.—*Rabbits Immunised per os.*

Rabbit.	Weight.	Test-dose.	Result.
No. 111.	1640 grs.	0·7 c.c. "soluble" toxin intravenously.	No symptoms. Survived.
No. 112.	1440 grs.	Ditto.	Paralysis of hind legs on 2nd day. Died 3rd day. <i>Lung</i> : Congestion and hæmorrhage. <i>Liver</i> : Coccidiosis. <i>Small intestine</i> : Edema, injection of submucosa. <i>Large intestine</i> : Slight edema. <i>Spinal cord and medulla</i> : Degenerative change in nerve-cells, hæmorrhage.
No. 113.	1900 grs.	1·4 c.c. "soluble" toxin intravenously.	Paralysed on 2nd day. Died 3rd day. <i>Lung</i> : Congestion and hæmorrhages. <i>Spleen, liver and kidney</i> : Congestion. <i>Small intestine</i> : Injected. Edema. <i>Large intestine</i> : Injection of submucosa. Edema. <i>Appendix</i> : Injected. <i>Spinal cord</i> : Hæmorrhages in grey substance and degenerative change in nerve-cells. <i>Medulla</i> : As spinal cord.
No. 114.	1300 grs.	Ditto.	No symptoms. Survived. Killed and examined. <i>Lung, Liver and Kidney</i> : Nil. <i>Spleen</i> : Congestion. Cells of germ centres swollen. <i>Small intestine</i> : Injection and edema of tunica propria, some small capillary hæmorrhages. <i>Spinal cord and medulla</i> : Nil.
No. 115.	1760 grs.	20,000 $\times$ 10 <sup>6</sup> washed bacilli killed at 80° C. injected intravenously.	No symptoms. Survived. Killed and examined. <i>Lung</i> : Nil. <i>Spleen</i> : Severe congestion of pulp. <i>Small intestine</i> : Injection of tunica propria. <i>Spinal cord and medulla</i> : Nil.
No. 116.	1760 grs.	Ditto.	No symptoms. Survived. Killed and examined. Similar changes to those found in Rabbit 115.

C.—*Control Rabbits.*

No. 129.	1060 grs.	0·7 c.c. "soluble" toxin intravenously.	Found dead on morning of 2nd day. <i>Lung</i> : Congestion and hæmorrhage. <i>Spleen</i> : Severe congestion. <i>Liver</i> : Congestion and hæmorrhage. <i>Pancreas</i> : Hæmorrhages. <i>Kidney</i> : Congestion. <i>Small intestine</i> : Injection and edema of submucosa, with eosinophile infiltration. <i>Appendix</i> : Eosinophile infiltrate. <i>medulla</i> : Hæmorrhages. <i>Spinal cord</i> : Injection of grey matter.
No. 130.	1200 grs.	1·4 c.c. "soluble" toxin intravenously.	Found dead on morning of 2nd day. Post-mortem findings as in Rabbit 129.
No. 137.	1900 grs.	20,000 $\times$ 10 <sup>6</sup> washed bacilli killed at 80° C. injected intravenously.	No symptoms. Survived. Killed and examined. <i>Lung, liver, kidney</i> : Nil. <i>Spleen</i> : Enlarged. <i>Small intestine</i> : Injected. <i>Large intestine</i> : Nil. <i>Spinal cord and medulla</i> : Nil.

The titre of agglutinins for *B. dysenteriae* (Shiga) in the serum of each of these rabbits was estimated ten days after the third immunising dose of vaccine. The suspension employed was made from a 24-hour agar culture and contained 2000  $\times$  10<sup>6</sup> bacilli per c.c. Results were read after 4 hours at 37° C. and 24 hours at room temperature.



TABLE X.

A.—Rabbits injected subcutaneously with carbolised vaccine.

Rabbit . . .	No. 102	No. 103	No. 104	No. 105	No. 106	No. 107
Agglutinin titre for <i>B. dysenteriae</i> (Shiga)	1/80	1/160	1/160	1/320	1/320	1/320

B.—Rabbits immunised *per os*.

Rabbit . . .	No. 111	No. 112	No. 113	No. 114	No. 115	No. 116
Agglutinin titre for <i>B. dysenteriae</i> (Shiga)	1/10	<i>Nil</i>	<i>Nil</i>	1/40	1/40	<i>Nil</i>

From the experiment given in Table IX it will be seen that a certain degree of immunity can be produced in rabbits to the readily soluble fraction of dysentery toxin (so-called exotoxin) by both methods of immunisation. As to the degree of immunity in the two series A. and B. there is little to choose. Certainly those rabbits which had received subcutaneous inoculations of a carbolised vaccine showed more extensive pathological changes in the intestine and hæmorrhages were more numerous, which fact might, at first sight, be taken as evidence of some local immunity in the intestinal wall produced by the ingestion of the heat-killed vaccine. A closer inspection of the evidence, however, reveals the fact that the rabbits in question, Nos. 104, 105 and 106, were all infected with coccidia, so that it was impossible to consider these hæmorrhages as necessarily due to the test dose of toxin. It was therefore impossible to demonstrate conclusively any local intestinal immunity. Those rabbits which died in both series A. and B. succumbed to the nerve toxin. The data given in Table X show that the subcutaneous administration of vaccine is productive of a higher antibody titre than the oral immunisation.

As the experiment above detailed did not yield conclusive data of comparative value, it was decided to repeat in the hope that a definite conclusion might be arrived at. Two series of rabbits, six animals in each series, were immunised as before, one lot by subcutaneous inoculation of a carbolised vaccine, the other by the oral administration of heat-killed bacilli. In this case total leucocyte counts were made daily on each rabbit, both during the immunisation and after the administration of the test dose of toxin, and the temperature of each animal was similarly recorded. The object of this was to obtain some idea of the influence of these two methods of immunisation on the rabbits, and also to obtain further information as to the comparative effect of the test dose of toxin on the animals of these two series.

It has been pointed out by Lüdke (1911) amongst others that a leucocytosis occurs after the injection of dysentery toxin. Space would not permit of the inclusion in this paper of these daily observations on the leucocytic count and body temperature. Generally speaking there was an increase in the total white count and a rise in temperature of 1°–3° C. following each immunising dose. These changes were more severe in those rabbits receiving the sub-

cutaneous inoculations; apparently the ingestion of the vaccine is productive of less general reaction. The observations made four hours after the inoculation of the test dose of soluble toxin intravenously showed a rise in temperature of 2–3° C. accompanied generally with a fall in the total leucocyte count. This leucopenia occurred in all six of the rabbits immunised *per os*, whereas two of the six animals which had been inoculated subcutaneously showed no fall in the leucocyte count, and in two others only a moderate degree of leucopenia resulted.

The figures bearing on this point are here annexed :

*Orally Immunised Series: Total Leucocytes.*

Before test dose intravenously	11,567	11,898	10,245	9,915	9,135
Four hours afterwards	1,718	5,783	2,223	4,296	1,718

*Series immunised by the subcutaneous method.*

Before test dose intravenously	9,915	8,758	11,567	9,414	8,265
Four hours afterwards	1,720	5,453	11,563	5,783	18,930

TABLE XI.

Series A.—Rabbits immunised *per os*. (Three doses of  $\frac{1}{2}$  of a Roux bottle culture.)

Rabbit.	Test-dose.	Symptoms.	Result.
No. 138.	2.0 c.c. "soluble" dysentery toxin.	Diarrhœa. Paralysis of fore and hind legs.	Died, 2nd day.
No. 139.	Ditto.	No symptoms.	Survived.
No. 140.	"	Diarrhœa.	Died, 2nd day.
No. 141.	"	Diarrhœa. Paralysis of fore and hind legs.	Died, 4th day.
No. 142.	"	Diarrhœa. Incontinence of urine. Paralysis.	Died, 3rd day.
No. 143.	"	Diarrhœa. Paralysis of fore and hind legs. Incontinence of urine.	Died, 4th day.

Series B.—Rabbits immunised by subcutaneous inoculation. (Three doses 50, 100 and 100 × 10<sup>6</sup>.)

No. 144.	2.0 c.c. "soluble" dysentery toxin.	Paralysis of hind legs. No diarrhœa. Incontinence of urine.	Died, 4th day.
No. 145.	Ditto.	Paralysis of fore and hind legs. No diarrhœa. Recovered 7th day.	Survived.
No. 146.	"	Paralysis of fore and hind legs. Slight diarrhœa on 3rd day.	Survived.
No. 147.	"	Paralysis of hind legs. Diarrhœa.	Died, 3rd day.
No. 148.	"	Paralysis of hind legs. Diarrhœa.	Died, 4th day.
No. 149.	"	No symptoms.	Survived.



Five control animals were inoculated with varying doses of the soluble toxin with the following results :

Rabbit.	Weight.	Dose of toxin.	Result.
No. 155.	1350 grs.	0.1 c.c.	No symptoms. Survived.
No. 154.	1400 grs.	0.5 c.c.	Paralysed. Died 2nd day.
No. 153.	1500 grs.	1.0 c.c.	No symptoms. Survived.
No. 156.	1390 grs.	2.0 c.c.	Died, 24 hours after inoculation.
No. 157.	1440 grs.	2.0 c.c.	Paralysis of hind quarters and diarrhoea for two days. Recovered.

In Table XI the results of this experiment are detailed, the intravenous test dose of the soluble toxin being fixed at 2 c.c. Without reference to the unimmunised controls, which at the same time received doses varying from 0.1 c.c. to 2 c.c. (*bis*), the results would indicate a considerable superiority of subcutaneous over oral immunisation, there being in the one series three survivals out of six, and in the other, one only. If, however, recovery without symptoms be taken as the criterion of complete immunity there would appear to be only one such in each series. As had happened in a previous experiment when attempt was made to determine the minimum lethal dose of the soluble toxin, aberrant results occurred with the control animals of this experiment also, and probably, on the whole, it would be safest to conclude from the evidence available that solid active immunity to the soluble dysentery toxin is difficult and capricious by whatever method it is sought. No clear-cut evidence was obtained of an immunity similar to that which was realised in previous work when the test dose consisted of living dysentery bacilli.

The action of this soluble dysentery toxin intravenously administered has some points of resemblance with that of the so-called "shock" toxins, against which it is difficult in small animals to secure protection by active immunisation with killed vaccines, however administered.

#### ANTI-BODY RESPONSE TO ORAL IMMUNISATION.

The anti-body response to the ingestion of a dysentery vaccine was also investigated, the agglutination titre for *B. dysenteriae* (Shiga) of the serum of each rabbit of Series A. being determined one week after each dose of vaccine. The sera were collected and kept in the cold room and all tested together with the same suspension, which was made from a 24-hour agar culture and contained  $2000 \times 10^6$  bacilli per c.c. The results are recorded in Table XII.

From Table XII it will be seen that the agglutination titre for *B. dysenteriae* (Shiga) rises appreciably with successive ingestions of bacilli. This is of interest, because Besredka's findings were entirely opposed to these. In his experiments he noted the appearance of agglutinins in the serum following the first ingestion of bacilli, after which they gradually disappeared, the subsequent immunising doses of the vaccine giving rise to no anti-body response. He

cites this observation in support of his contention that oral immunisation has produced an increased *local* resistance in the intestinal mucosa.

TABLE XII.

Time of observation.	Rabbit—				
	No. 144.	No. 145.	No. 146.	No. 147.	No. 148.
One week after 1st dose.	—	1/80	1/10	1/20	1/20
One week after 2nd dose.	1/10	1/80	1/20	1/80	1/20
One week after 3rd dose.	1/40	1/320	1/40	1/80	1/160

## DISCUSSION.

The main object of these experiments, viz. the determination of the degree of local immunity in the intestinal mucosa produced by the ingestion of dysentery bacilli, was not achieved. The failure arose from two causes. First of all, no strain of *B. dysenteriae* (Shiga) was found which, when inoculated into rabbits, gave rise to intestinal lesions to the exclusion of other pathological changes, and secondly, all attempts to separate endotoxin from exotoxin, on the lines of Olitsky and Kligler's work, proved fruitless. Certainly a portion of the toxin is readily dissolved out of the bodies of the dysentery bacilli. It was only necessary to suspend an agar culture in normal saline and to remove the bacilli by centrifugalisation to obtain a highly toxic supernatant fluid. This soluble fraction, however, produced the same lesions in the rabbits as the whole bacilli—changes not wholly confined to the central nervous system—and when heated for one hour at 80° C. all toxicity was lost. It must be admitted that the bacilli, even after washing ten times, still retained some of their toxicity. But when bacilli so treated were submitted to a temperature of 80° C. for one hour their toxicity was practically destroyed. Out of six rabbits inoculated with washed and heated bacilli, only one died—the one which received  $20,000 \times 10^6$  bacilli—and though this animal showed hæmorrhages in the intestinal mucosa and other viscera, the presence of a heavy coccidiosis infection rendered it doubtful whether these lesions were to be placed to the account of the dysentery toxin or not.

## CONCLUSIONS.

(1) The toxin of *B. dysenteriae* (Shiga) affects principally the central nervous system (medulla and spinal cord) in rabbits. At the same time it acts upon the capillary circulation generally with the production of congestion and hæmorrhage in the various viscera.

(2) Exposure to a temperature of 80° C. for one hour markedly reduces its toxicity, especially for the central nervous system.

(3) It has been impossible to effect a separation between exotoxin and so-called endotoxin on the lines of Olitsky and Kligler.



(4) Though there is some indication in the foregoing experiments that oral immunisation produces a local immunity in the intestinal wall, this point remains undecided.

(5) The oral administration of the Shiga vaccine gives rise to less general reaction than does its inoculation subcutaneously.

(6) The antibody titre of the serum of rabbits immunised *per os* is appreciably raised by repeated ingestions.

(7) Attempts to secure, by subcutaneous and oral administration of killed vaccines, a solid active immunity against the potent soluble toxin of *B. dysenteriae* (Shiga) intravenously administered, have not been attended with the striking success realised in previous work when active immunity was sought to the live bacilli similarly administered. Consequently, comparative estimates of the value of oral and subcutaneous methods in this connection are not at present possible.

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## EXPERIMENTS EMPLOYING A QUANTITATIVE METHOD IN A STUDY OF THE D'HÉRELLE PHENOMENON.

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THE phenomenon of d'Hérelle consists, in general terms, of changes in bacterial cultures initiated by some substance of obscure nature that has been obtained from most diverse sources. One of the striking changes is lysis, indicated by clearing of a bouillon culture. Lysis can be reproduced in series, thus giving the impression that the substance causing it has the power of growth. The exact nature of this substance is a controversial point, and a review of the literature has been published in another place (Maitland, 1922).

Throughout this paper the substance will be termed the "active substance." A quantitative method for estimating the strength of a sample of active substance is described. It is pointed out that increase of active substance in a bouillon culture can occur without lysis; in fact it appears to bear no relation to changes in bacterial content of the culture in which it is increasing. The quantitative method is also applied to the elucidation of other problems.

### PREPARATION OF SAMPLES OF ACTIVE SUBSTANCE.

Seven samples were isolated from fæces of hospital patients. Four had infection with *B. paratyphosus* B, two with *B. typhosus*, and one had acute appendicitis followed by portal pyelophlebitis and liver abscesses which yielded *B. mucosus capsulatus*.

The technique was to add about 0.5 c.c. fæces to 50 c.c. bouillon (pH 7.8-8.0) and incubate at 37° C. overnight. The culture was filtered through infusorial earth and a Mandler filter. One cubic centimeter of this filtrate added to a young bouillon culture of an appropriate organism resulted in the appearance of active substance. All samples acted on several organisms and produced typical effects in successive cultures.

Table I shows the origin of the samples, the organisms acted upon, and indicates in a general way the amount of action.



TABLE I.

Name of patient.	Timbers.	Collins.	Aitken.	Rotz.	Gibson.	Walker.	McPhee.
Patient's infection	<i>B. paratyphosus</i> B.	<i>B. paratyphosus</i> B.	Appen- dicitis. Liver ab- cesses.	<i>B. paratyphosus</i> B.	<i>B. paratyphosus</i> B.	<i>B. typhosus</i> B.	<i>B. typhosus</i> B.
Organisms acted upon {							
<i>B. coli</i>	0	0	0	+	0	0	0
<i>B. paratyphosus</i> A.	+	+	+	+	0	0	0
<i>B. paratyphosus</i> B.	++	++	++	++	0	+	+
<i>B. dysenteriae</i>	++	++	++	++	+	+	+
<i>B. typhosus</i>	0	0	0	+	0	0	0

The organisms acted upon were all from stock cultures, and were the only ones employed for the experiments of this paper.

#### MEASUREMENT OF THE STRENGTH OF A SAMPLE OF ACTIVE SUBSTANCE.

The method of measurement devised for these experiments is based on the graded appearances illustrated in Fig. 1.

When the strength of any sample is to be determined six tubes are set up. The first contains active substance, the others bouillon. A dilution of 1 in 10 is made in the second tube, 1 in 100 in the third, continuing to 1 in 100,000 in the sixth. Six agar slants are inoculated, each with one loopful from a tube of active substance and a loopful of a young bouillon culture of the organism in association with which that sample of active substance had been produced. After incubation overnight the strength of the sample will be indicated by comparing each tube with the graded series in Fig. 1. For convenience in recording results the gradations have been designated by numbers. Thus the strength of a sample might be indicated by 8 7 6 4 2 1.

The method described does not depend on any one dilution, but takes into account all six tubes. It does not depend upon any theory as to the nature of active substance. It has given consistent readings with the same sample. The expression of strength is not absolute, but it has proved to be consistently comparative. It seems to be, therefore, the nearest approach to accurate measurement that can be obtained, in the absence of exact knowledge as to the nature of the substance being measured. As such it has been used to make a quantitative study of some aspects of the phenomenon under discussion.

#### THE RATE OF INCREASE OF ACTIVE SUBSTANCE IN BOUILLON CULTURES.

For the investigation of this point active substance derived from Timbers and *B. dysenteriae* was employed.

To each of several identical tubes of young bouillon culture was added an amount of active substance known by previous determination to give a reading of about 3 0 0 0 0 0. The tubes were placed at 37° C. From time to time one was filtered through a Mandler filter and the strength of the filtrate measured. Thus a number of readings were obtained giving the strength of samples at various times. A control of active substance in bouillon in every instance showed no increase.

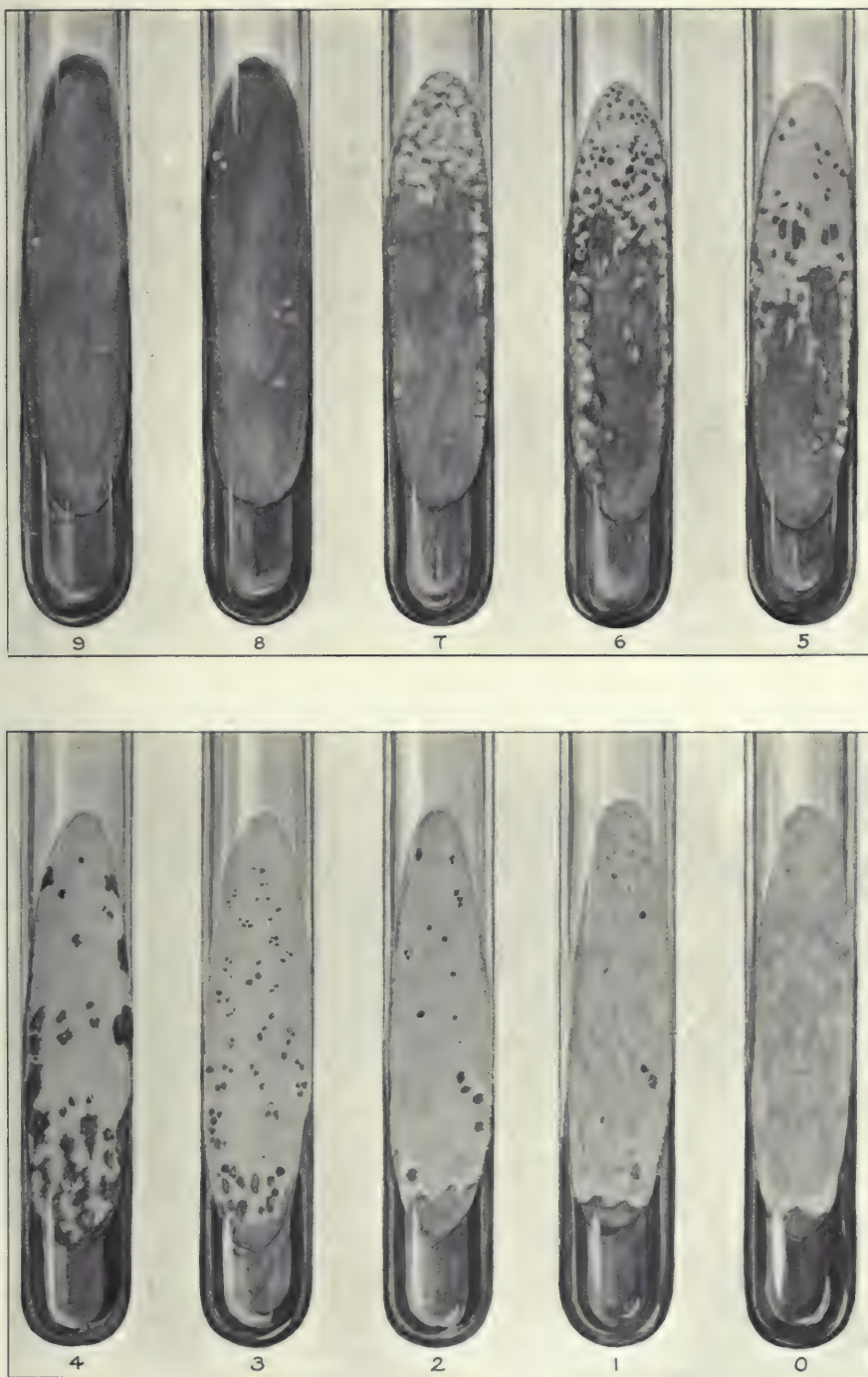


FIG. 1.

Graded appearances on agar slants inoculated with *B. dysenteriae* and falling doses of active substance after 24 hours' incubation. Tube No. 9, showing complete inhibition of growth, corresponds to a full strength sample of active substance, and the decreasing numbers to progressively increasing dilutions. In tube No. 0 the dilution is so great that the bacterial growth appears normal. All tubes were made from the same sample of active substance and *B. dysenteriae*.





Some results are given to illustrate this increase. Timbers' active substance was transferred in series on *B. dysenteriae*, and therefore had always acted on that organism.

TWO-HOUR CULTURE.							TWENTY-HOUR CULTURE.						
Time after addition of active substance.		Readings.					Time after addition of active substance.		Readings.				
10 min.	2	0	0	0	0	0	10 min.	1	1	0	0	0	0
1 hr.	3	1	0	0	0	0	1 hr.	1	0	0	0	0	0
2 hrs.	7	6	5	1	1	1	2 hrs.	2	0	0	0	0	0
3 hrs. 45 min.	8	8	6	4	2	1	3 hrs. 45 min.	6	5	1	1	0	0
5 hrs. 45 min.	9	8	8	5	4	1	5 hrs. 30 min.	8	7	5	3	1	1
7 hrs.	8	8	7	5	3	1	8 hrs. 15 min.	8	8	4	1	1	0
22 hrs. 30 min.	9	8	8	7	6	3	11 hrs. 45 min.	8	8	4	3	1	1
49 hrs. 30 min.	9	8	7	7	3	1	25 hrs. 30 min.	8	8	7	5	3	1
9 days	9	9	8	6	6	1	7 days	8	8	6	5	2	1

TWENTY-FOUR-HOUR CULTURE.						
Time after addition of active substance.		Readings.				
1 hr. 10 min.	2	0	0	0	0	0
3 hrs. 30 min.	8	8	6	5	2	1
5 hrs.	9	7	5	4	2	1
7 hrs. 30 min.	8	8	6	4	1	0
4 days	8	8	7	4	1	1

These readings demonstrated that increase does occur. The method affords, therefore, a means of detecting changes in strength of active substance in any experimental circumstances.

It was also shown that the curve of increase had three phases. During the first hour, or occasionally longer, there was no increase. This was followed by a period of rapid increase. Generally this occupied the second and third hours, during which the active substance increased to about ten thousand times its original strength. During the fourth hour the rate of increase slackened. By the fifth hour it appeared to be established at its maximum. From that time the curve entered the third phase of equilibrium, which lasted as long as readings were made, up to nine days.

#### FACTORS AFFECTING THE INCREASE OF ACTIVE SUBSTANCE IN BOUILLON CULTURES.

It was found by measurement that active substance did not increase in a saline suspension of living bacteria, from an eighteen-hour bouillon culture, washed free from all growth products and accessory substances. Timbers' active substance and *B. dysenteriae* were used. It was evident therefore that three factors were necessary for increase: (1) a small amount of active substance itself, (2) living bacteria, (3) some accessory factor.

Various substances were then tested to determine if they contained an accessory factor. Small amounts were added to active substance in a saline suspension of washed bacteria and the method of measurement applied to detect increase.



It was found that bouillon (prepared with "Difco" peptone) itself acted as an accessory factor and that the minimum effective amount lay somewhere between 1 per cent. and 0.1 per cent. Active substance did not, however, increase in bouillon alone.

The following readings serve to illustrate:

*B. dysenteriae* from a 24-hour Bouillon Culture: Timbers' Active Substance.

	Time after addition of active substance.	Readings.					
Saline suspension + 5 per cent. bouillon	5 hrs.	8	7	6	2	0	0
	8 hrs.	8	6	6	3	2	0
	24 hrs.	9	7	5	5	2	0
Saline suspension + 1 per cent. bouillon	5 hrs.	6	3	2	0	0	0
	8 hrs.	8	7	4	2	2	0
	24 hrs.	8	7	5	4	1	0
Saline suspension + 0.1 per cent. bouillon	5 hrs.	2	1	0	0	0	0
	8 hrs.	2	0	0	0	0	0
	24 hrs.	2	0	0	0	0	0
Control, active substance + saline suspension alone	24 hrs.	2	1	0	0	0	0

The addition of bacterial growth products to bouillon did not alter its effectiveness. That is to say, a Berkefeld filtrate of an eighteen-hour bouillon culture of *B. dysenteriae* had the same effect as bouillon alone.

Meat infusion prepared as in making bouillon but without salt or peptone gave the same result as bouillon. Hæmoglobin appeared not to be essential. Extracts were made of sheep's erythrocytes and defibinated sheep's blood, but no active preparation was obtained. The extracts were made by adding 2.5 per cent. washed erythrocytes or 5 per cent. of defibrinated blood to distilled water. The mixtures were heated at 50°C. for 30 minutes, and boiled for 15 minutes. At this stage they were inactive. These permanently turbid solutions were then precipitated by adding NaCl. The supernatant fluid was autoclaved at 15 lb. for 30 minutes and gave a yellowish tinged fluid that was also inactive.

Ascitic fluid free from hæmoglobin was effective, as was also human citrated plasma.

The third factor was also found in potato. A piece from the centre of a potato was removed aseptically, minced, and extracted in normal saline for 36 hours or longer. This extract, bacteria free, permitted increase.

THE RELATION OF THE CURVE OF INCREASE OF ACTIVE SUBSTANCE  
IN A BOUILLON CULTURE TO VARIATIONS IN THE NUMBER  
OF ORGANISMS.

If increase in active substance depends on bacterial division it should be possible to demonstrate some relation between this increase and the bacterial increase. One cannot, however, use the growth rate in a culture that contains active substance on account of the factors of lysis and inhibition. Just how these might operate would be difficult to predict; the observation of many

cultures suggests that it is not always in the same manner. In some cases increase is accompanied by clearing or by inhibition, but there may be increase with very little change in the bacterial content.

It can be demonstrated that the curve of increase has no apparent relation to the growth curve in a parallel culture without active substance. Employing a saline suspension of washed living bacteria plus 1 per cent. bouillon there was increase according to the readings cited, but the bacteria in a parallel tube at most doubled in number. The method described by Gates (1920) was used to estimate the number of organisms. It was found, also, that active substance increased equally in four- to six-hour cultures and in twenty-four-hour cultures, in which the growth rate was dissimilar at the time active substance was added.

Therefore it appears that active substance can increase some ten thousand-fold while the number of bacteria in a parallel culture is doubling. There is no reason for supposing that growth would be more rapid in the tube with active substance. On the other hand the effect of active substance may be quite complex, and variation of the total bacterial content may be the sum of destruction, inhibition and stimulation proceeding simultaneously. It is possible also that bacterial metabolism may be affected independently of bacterial division. Although the absence of apparent relation between increase and the growth curve in a parallel culture is of interest in itself, it does not essentially advance the problem of the interaction of active substance and bacteria. The fact that increase may coincide with varying changes in the total bacterial content of the culture suggests that the interaction is probably the sum of several effects proceeding simultaneously.

#### THE EFFECT ON THE LYTIC AND INHIBITING QUALITIES OF ACTIVE SUBSTANCE OF THE ORGANISM IN ASSOCIATION WITH WHICH IT IS PRODUCED.

If increase of active substance is the result of bacterial metabolism, and since increase will occur with more than one organism, it might be supposed that the qualities of a sample would be influenced by the organism in association with which it is produced.

This possibility was examined by the following experiment. A filtrate from a culture of fæces (Walker) was found to yield active substance when added to *B. dysenteriae* and *B. paratyphosus B*. The quality of the sample obtained by filtering the first culture of *B. dysenteriae* was subjected to the method of measurement with two organisms, *B. dysenteriae* and *B. paratyphosus B*. This sample was allowed to increase through twelve cultures of *B. dysenteriae* and measured again. If any change in the lytic or inhibiting qualities had occurred there should have been some difference in the readings. All the readings were, however, identical.

Similarly, the filtrate from the first culture of *B. paratyphosus B* was passed through twelve cultures of *B. paratyphosus B* and measured with both organisms before and after. All these readings were identical. Also, they were identical with those of the other sample.

Thus the lytic and inhibiting qualities of a freshly isolated sample of active substance were not altered by allowing it to increase repeatedly in association with different organisms. If these organisms actually did produce the increase

then *B. dysenteriae* and *B. paratyphosus B* must each have produced a sample with the same qualities.

#### CONCLUSIONS.

The rate and amount of increase of active substance in a culture can be measured.

The curve of increase has three phases, an early period of very slight increase, a comparatively short period of rapid production, and a stationary period commencing when an equilibrium has been reached at about five hours and lasting for at least nine days.

Three factors are necessary for increase: (1) A small amount of active substance, (2) living bacteria, (3) an additional factor.

The increase of active substance in a bouillon culture does not coincide with any constant alteration in the bacterial content of the culture in which it is being produced. Clearing does not always occur, and there may be increase in the total number of bacteria.

The lytic and inhibiting qualities of a sample of active substance were not altered by causing it to increase repeatedly in association with two different organisms. If the organisms themselves produced the active substance then each (*B. dysenteriae* and *B. paratyphosus B*) must have produced a sample with the same qualities.

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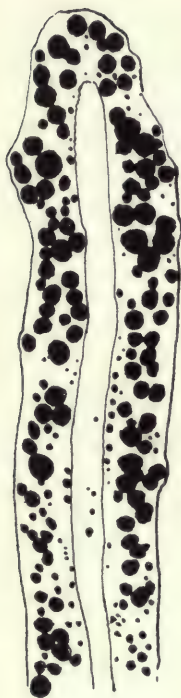


FIG. 1.



FIG. 2.



FIG. 3.

Low power camera lucida drawings of villi stained with osmic acid. Fig. 1 shows absorption by drops. From a rat fed on a vitamin-free diet. Fig. 2 shows absorption by streams. From a rat fed on a diet containing vitamins A and B. Fig. 3 shows absorption by drops at the apex of the villi and streams at the base. From a rat fed on a diet containing vitamin B, but not A.

+ VITAMIN B.

NO VITAMINS



FIG. 4.—High-power camera lucida drawing of intestinal epithelium (duodenum) of two rats. The one had received a vitamin-free diet, the other a diet containing vitamin B.

## VITAMINS, EXPOSURE TO RADIUM AND INTESTINAL FAT ABSORPTION.

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NUMEROUS descriptions and figures are given in the literature of the histological appearances of the absorption of fat by the epithelial cells of the small intestine in mammals. They all show as the first stage an appearance of droplets of fat between the free border and the nucleus. These increase in size and may fill the whole cell. How these fat-droplets leave the cell again is a question which has given rise to a good deal of discussion, but no satisfactory solution of the problem has as yet been found. After they have left the epithelial cell the fat-droplets find their way into the central lacteal of the villi, and in this transport the lymphocytes, according to Schafer (1912), play an important part. We shall call this mode of fat absorption "absorption by drops." Schafer also states that puppies and kittens fed on milk show a different type of fat absorption: "darkly stained streaks may be seen (in osmic preparations) extending from the interepithelial spaces to the borders of the central lacteal." This type of fat absorption when the fat is in a much more finely divided form we shall call "absorption by streams." So far as we have been able to find, it is mentioned only briefly in Schafer's text-book and no figure of it appears to exist. The conditions governing the two types of fat absorption are not known. During the study of the lymphoid atrophy following radiation and vitamin B deficiency it was found that these two types of fat absorption can be produced at will in the same animal. The object of the present paper is to report the main facts observed.

Rats were the animals experimented upon. They were kept without food for 20 hours. They were then fed artificially at a given time by passing a narrow glass tube down the throat through which the food was introduced. The animals to be compared were fed at the same time and killed some hours later at the same time. The number of hours varied in different experiments from  $2\frac{1}{2}$  hours to 7 hours. Pieces of different parts of the small intestine exactly equidistant from the pylorus were cut out, fixed in formalin, and then transferred to a solution of 1 per cent. osmic acid in 1 per cent. chromic acid. The tissue was examined in paraffin section.

In the first set of experiments two batches of animals were fed, the control set on standard diet and olive oil, the other set B with standard diet, olive oil and vitamin B, given in the form of "marmite."



The standard vitamin-free diet consisted of starch, purified casein and salt mixture.

In set B in which vitamin B was present the fat was absorbed by streams, in some cases by drops at the apices of the villi and by streams at their sides. In the control set which had no vitamin the fat was absorbed entirely by drops. If the fat is absorbed by streams the central part of the cell between the nucleus, which is usually centrally placed, and the free border is free from fat-droplets, and a stream of very finely divided fat-droplets is seen running through the cell on either side of the nucleus. Fine fat-droplets are also present in abundance in the reticular tissue in the centre of the villi. We wish to emphasize that the stream of droplets in our preparations is within the epithelial cell: we have not seen the passage of fat-droplets between the cells described by Reuter (1902). If absorption is by drops, large globules of fat accumulate in the central part of the cell between the free border and the nucleus, which is frequently pushed towards the basement membrane. Very few fat-droplets are to be seen in the reticular tissue in the centre of the villus, and when present they are relatively large. The difference between the two appearances is so striking that a glance with the low power at the preparation enables one to tell with certainty whether the animal had been fed on a vitamin-rich diet or a diet free from vitamins.

In a second set of experiments cod-liver oil was used instead of olive oil, so that the animals received an ample supply of vitamin A. When vitamin B was added the fat was absorbed entirely in the form of streams. When vitamin B was omitted and only the fat-soluble vitamin A was present the absorption took place in drops at the apex and a few streams at the sides. It should be specially noted that these effects were observed in normal rats which had not been subjected to any vitamin deficiency previous to these experiments. The effect on fat absorption is therefore an immediate one.

The effect of radiating animals was next investigated. In order to determine how the great reduction in the number of circulating lymphocytes affects fat absorption, animals received a large dose (0.66 rads) of screened radiation (0.1 mm. lead). No fat absorption at all occurred, even in the presence of vitamin B and A. When 0.28 rads was given, only absorption by drops occurred whether or not vitamins were present. When 0.09 rads was the dose applied, then olive oil with vitamin B gave only drops, and cod-liver oil containing vitamin A with the further addition of vitamin B only drops and slight streams at the bases of the villi.

That almost all previous observers have found only the fat absorption by drops explains itself readily from the fact that when details are given the fat used for feeding was either bacon fat or lard or olive oil. These materials are free from the water-soluble vitamin B and contain little if any fat-soluble vitamin A. It is significant that the absorption of fat by streams described by Schafer was seen in animals fed on milk which contains both vitamins. We have noted that in the rats receiving a vitamin-free meal relatively large masses of fat are sometimes seen lying within the lumen of the intestine, between the opposing villi. This suggests that the digestion of fat is less complete or less rapid in the absence of vitamins, and that vitamins may have a stimulating action on the digestive processes. Voegtlin and Myers (1919)

have shown that food rich in vitamin B contains substances which stimulate the secretion of bile and of pancreatic juice. And although their further contention that vitamin B and secretin are identical has been disproved by the work of Anrep and Drummond (1921), the actual observations of the last-named authors confirm the important and significant fact that the activity of the liver and pancreas can be stimulated by the presence of vitamin B in the food.

The effect of radiation is a striking confirmation of Schafer's views on the importance of the lymphocytes in fat absorption. In fact their function appears to be even more important than the mere transport of fat which he assigned to them. Their presence or absence seems to determine the physiological condition of the epithelial cells even more powerfully than the presence of vitamins.

There is, as our figures show, an essential difference with reference to the functional activity of food absorption between the state of a cell absorbing fat by drops, and the state of a cell where a stream of very finely divided fat runs through the cell on either side of the nucleus as if the cell were canalised.

The problem how exactly these various facts are related to each other and what secondary effects they may produce in the bacterial flora of the intestine is a matter for further investigation. One factor we can definitely exclude: experiments with coloured food show that the rate of passage of food through the stomach and small intestine is not delayed at once when vitamins are withheld from the food. There is no intestinal stasis in that sense. A deficient absorption of fat is in itself not an adequate explanation of the marasmus which results from a deficiency of the water-soluble vitamin or from exposure to radium, since animals can live on a diet free from fat. But if we take the difference in the absorption of fat described in this paper as an indicator of difference in the functional state of the intestinal epithelium in regard to the absorption of food, we can draw the general conclusion that the vitamins A and B, particularly the latter, produce a definite stimulating action on the processes of intestinal digestion and absorption. The presence of lymphocytes in the intestine is an even more important factor. This confirms the conclusion expressed by us in a previous paper (Cramer, Drew and Mottram, 1921), that the marasmus resulting from withholding the water-soluble vitamin from the diet is due to an impaired absorption and assimilation of food from the intestine.

The radium used in this investigation was a loan from the Medical Research Council.

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## INDICATORS FOR CULTURE MEDIA CONTAINING VARYING ACIDS AND BUFFERS.

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WHEN the final pH of a bacterial culture is ascertained, the determination has often to be made in the presence of various acids produced during growth, such as lactic, acetic, succinic, etc. Similarly, in estimating the pH values of pathological fluids associated with infective or other agents, the dissociation products and buffer influences of carbonic, sulphuric, acetic and phosphoric acids have to be taken into account.

During an investigation in which dilute solutions of typical examples of the various types of acids were introduced into culture media prior to insemination with bacteria, it was recognised that a wider experience with colour indicators was called for, more especially with regard to the behaviour of the indicators in mixtures containing neutral salts or solutions of weak acids.

Discussing the problems involved with Prof. J. W. McBain, of our University Department of Physical Chemistry, I was offered the services of one of his "Honours" students, Miss M. Kieser, for the control of our colorimetric estimations by E.M.F. determinations. She has carried out this part of the work with great care, and is responsible for the electrical measurements.

In addition to the object of ascertaining the limits of colorimetric work, it was considered worth while to examine in detail the buffer content of the media employed.

### METHOD.

A large batch of casein sugar-free broth (Hall, 1918) or other medium was prepared. This was made neutral to phenol-phthalein, and then brought to the required pH by the addition of the necessary amounts of the several normal acid solutions, and a colorimetric estimation made. Immediately this had been done, a sample was taken by Miss Kieser, and the E.M.F. determined. After the E.M.F. reading was taken, an additional colour test was carried out.

The acidity and alkalinity reserve was ascertained by a method similar to that of Brown (1921) and the buffer index calculated.

### BUFFER CONTENTS.

The variations of the buffer factors in different media cannot be neglected, especially when a rapid or large production of acidity is aimed at, or when it



is desired that the final pH shall be low. Some of our composite media protect bacterial substance from acids or salts, as for instance by the additions of calcium or other substances, while others fail to do so, and consequently are associated with less active metabolism and growth. This absorption or abstraction may also decrease or augment the differential manifestations of a bacterium, and accordingly alter its specific capacities. Similarly in making use of the action of cation, anion, osmotic or dissociation influences during growth, it is an advantage to standardise the reserve alkalinity and acidity, that is to say, the buffer index, of each batch of medium employed.

By this means the micro-organisms are provided with more stable conditions for developing their fullest activities and imitation of the "*in vivo*" surroundings is more nearly attained.

Quite apart from the question of accelerating or inhibiting the growth of particular organisms, Brown (1921) suggests that a medium with a low buffer index should be selected when rapid indications of fermentation are required, and one of high reserve alkalinity and buffer index when abundant growth and large acid production are desired. As a guide, therefore, the contents of Table I are submitted. The average amount of N/10 acid necessary to adjust the medium from phenolphthalein to phenol red neutrality is indicated in brackets as dilutions.

Thus  $\text{HNO}_3$  in the veal broth represents a dilution of N/10  $\text{HNO}_3$  in the total fluid medium of 1 in 14, in peptone 1 in 12, etc.

TABLE I.—*Comparative Buffer Indices of Media pH 7.6 and Equal Amino-Acids containing Dilute Acids.*

Acids.	Dissocia- tion per cent. at 18°- 25°C.	Lait-proto broth 1.8.	Veal broth 2.2.	Lait-proto broth + 1 per cent. peptone 1.9.	Veal broth + 1 per cent. peptone 2.55.	Bacto- peptone 3.3 per cent. 2.85.	Bacto- peptone 1.2 per cent. 1.95.	Meat extract 2.6.
Mineral:								
$\text{HNO}_3$ . . . .	94	1.9(14)	2.4(14)	1.95(14)	2.7(13)	3.1(12)	2.2(12)	2.8(13)
$\text{HCl}$ . . . . .	91	1.85(14)	2.3(14)	1.95(14)	2.7(13)	3.0(13)	2.15(13)	2.8(14)
$\text{H}_2\text{SO}_4$ . . . .	62	1.85(14)	2.5(14)	2.0(14)	2.8(13)	3.2(12)	2.2(12)	2.6(13)
Phosphoric . .	13.9	1.8(8)	2.4(8)	1.9(8)	2.6(8)	2.8(7)	1.8(7)	2.6(8)
Sat. monobasic:								
Formic . . . .	4.5	1.95(14)	2.5(14)	1.9(14)	2.7(13)	3.0(12)	2.2(12)	2.7(14)
Acetic . . . .	1.3	2.0(14)	2.5(14)	2.0(14)	2.9(13)	3.3(12)	2.2(12)	2.8(13)
Propionic . . .	1.2	1.9(14)	2.4(15)	2.0(14)	2.8(13)	3.1(12)	2.1(12)	2.7(13)
Butyric . . . .	1.1	1.95(14)	2.4(14)	1.9(14)	2.8(13)	3.1(12)	2.1(12)	2.7(13)
Sat. dibasic:								
Oxalic . . . .	9.0	2.0(14)	2.5(14)	2.0(14)	2.9(13)	3.0(12)	2.1(12)	2.7(13)
Monobasic hydroxy:								
Lactic . . . .	3.6	1.95(14)	2.4(14)	2.0(14)	2.8(13)	3.1(12)	2.2(12)	2.7(13)
Dibasic dihydroxy:								
Tartaric . . . .	13.9	1.95(14)	2.4(15)	2.0(14)	2.8(13)	3.0(12)	2.1(12)	2.7(13)
Tribasic hydroxy:								
Citric . . . .	15.2	1.9(14)	2.4(13)	2.0(14)	2.7(13)	3.0(12)	2.1(12)	2.7(13)

The findings show a slight buffer increase in the media containing the added acids; this is probably associated with the liberation of anions.

The effects of sterilisation and storage upon the buffer contents call also for inquiry. Taking all the findings into consideration, it may be stated that the changes are minimal so far as bacteriological requirements are concerned. An illustration is appended in Table II, the pH 6·8 being selected as a contrast to the 7·6 of Table I.

TABLE II.—*Effects of Storage on Buffer Indices in the Presence of Various Acids.*

Peptone water (amino-acid 40) (Hall, 1918).			
pH 6·8 adjusted with—	Before sterilisation.	After sterilisation.	Stored at 17° C. for 26 days.
HNO <sub>3</sub> . . . . .	2·25	2·37	2·37
HCl . . . . .	2·4	2·35	2·35
H <sub>2</sub> SO <sub>4</sub> . . . . .	2·6	2·54	2·54
Phosphoric . . . . .	5·0	4·89	4·89
Formic . . . . .	2·45	2·38	2·38
Acetic . . . . .	3·25	3·18	3·16
Propionic . . . . .	2·75	2·68	2·68
Butyric . . . . .	2·9	2·86	2·85
Oxalic . . . . .	2·75	2·67	2·65
Lactic . . . . .	2·0	1·96	1·96
Tartaric . . . . .	2·37	2·37	2·37
Citric . . . . .	2·75	2·74	2·73

It is common practice to add fresh blood, serum, tissue fluids or tissues to media in order to induce profuse bacterial growth. Frequently as much as 10 per cent. or more is added. McLeod and Wyon (1921) have shown that the growth-promoting power does not bear any ratio to the known vitamine contents, and suggest that it is a phenomenon of the colloid state. Whether it is in any way due to buffer changes seems worth the inquiry. Table III contains a few of the estimations to show that the buffer alteration is very slight when blood is employed and when the added acids are in such dilutions as fail to yield more than the lower limits of protein-acid combination, or to augment the viscosity. But both Tables II and III indicate the advantages to be gained by adjusting the various media used in a laboratory to a similar buffer index for purposes of comparative growths, and for maintenance of stock cultures. Equally, the preparation of media with buffer content suitable for rapid fermentation or slowed growth is evident.

TABLE III.—*Effect of Blood on Buffer Indices.*

	pH.	Reserve		Buffer index.
		Acidity.	Alkalinity.	
Lait-proto broth . . . . .	7·7	0·7	2·0	2·7
Lait-proto broth + 1/10 Blood . . . . .	7·6	0·75	2·25	3·0
Lait-proto broth + 1/10 Blood + 1/100 HNO <sub>3</sub> . . . . .	7·3	0·90	2·0	2·9
Lait-proto broth + 1/10 Blood + 1/200 HNO <sub>3</sub> . . . . .	7·5	0·80	2·4	3·2
Lait-proto broth + 1/10 Blood + 1/100 lactic . . . . .	7·3	0·90	2·0	2·9
Lait-proto broth + 1/10 Blood + 1/200 lactic . . . . .	7·4	0·80	2·2	3·0



## EXTENDED USE OF COLOUR INDICATORS.

The value of colorimetric readings is shown in Table IV. With practice it is possible to obtain close figures, even in the presence of all the types of acids employed. The results are, however, more likely to be less precise in mixtures when there are interacting substances and in the presence of sugars. The differences, nevertheless, are slight, and the error can be calculated.

Speaking generally, the E.M.F. finding is slightly in excess of the colour reading. This discrepancy may arise from a source similar to that which Evans (1921) describes in connection with the application of the E.M.F. to blood and other fluids containing carbonates or carbonic acid.

TABLE IV.—*Comparison of Colorimetric and E.M.F. Readings.*

Fluid media adjusted to 40 amino-acid and pH content with varying acids.

	Lait-proto broth.						Bacto-peptone.		Lait-proto broth + bacto- peptone.	Lait-proto broth + bacto- peptone + 1 per cent. glucose.
	Colour.	E.M.F.	Colour.	E.M.F.	Colour.	E.M.F.	Colour.	E.M.F.	E.M.F.	E.M.F.
HNO <sub>3</sub> . . .	3·3	3·1	5·2	5·5	7·5	7·9	6·8	6·7	6·8	6·2
HCl . . .	3·3	2·7	5·2	5·4	7·5	7·7	6·8	6·5	7·3	6·6
H <sub>2</sub> SO <sub>4</sub> . . .	3·3	2·9	5·2	5·4	8·0	8·0	6·8	6·8	6·6	6·7
Phosphoric . . .	3·3	3·5	5·2	5·6	7·5	7·4	6·8	6·8	6·8	6·7
Formic . . .	3·3	3·4	5·2	5·6	7·5	7·7	6·8	6·7	7·1	6·3
Acetic . . .	3·3	3·4	5·2	5·4	7·5	7·9	6·8	6·8	6·1	6·9
Propionic . . .	3·3	3·7	5·2	5·3	7·5	7·4	6·8	6·8	6·5	6·8
Butyric . . .	3·3	3·8	5·2	5·4	7·5	7·7	6·8	6·6	6·8	6·9
Oxalic . . .	3·3	2·5	5·2	5·5	7·5	7·5	6·8	6·8	6·9	6·9
Lactic . . .	3·3	3·1	5·2	5·6	7·5	7·8	6·8	6·8	7·0	6·6
Tartaric . . .	3·3	3·1	5·2	5·7	7·5	7·6	6·8	6·6	7·1	6·5
Citric . . .	3·3	3·0	5·2	5·4	7·5	7·7	6·8	6·8	6·9	6·7
Indicator . . .	Brom-phenol blue		Methyl red		Phenol red		Phenol red		Phenol red	Phenol red

## SUMMARY.

(1) Brom-phenol blue, methyl red and phenol red may be used as pH indicators for bacteriological media containing dilute quantities of certain typical mineral and organic acids.

(2) Buffer indices of media are slightly altered when small amounts of blood or of dilute acids are added. They are unaffected by storage.

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VARIATION PHENOMENA IN STREPTOCOCCI, WITH  
SPECIAL REFERENCE TO COLONY FORM, HÆMO-  
LYSIN-PRODUCTION, AND VIRULENCE.

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IN spite of the numerous elaborate researches which have appeared periodically for many years past, no generally accepted method of classifying streptococci has yet been arrived at. Marmorek (1895) found that the morphological and growth characteristics were far too variable to allow either as a basis for grouping. Since Bordet (1897) found hæmolysed blood in the hearts of animals dying as a result of streptococcal infections, and Schotmüller (1903) made a distinction between hæmolytic and non-hæmolytic strains when grown on blood-agar, a broad classification into these two varieties has received fairly general acceptance. Each of the groups, however, is so large that attempts to subdivide them have occupied the attention of many workers in recent years.

One method used for this purpose has been the differentiation of strains according to their action in the fermentation of sugars. Gordon (1905) and also Andrewes and Horder (1906) found their results in this respect sufficiently constant to be of value, and Winslow (1912) came to the same conclusion. Broadhurst (1913) found that fermentation results varied according to the media employed, and while Holman (1916) divided streptococci on this basis, he did not believe that the grouping was directly correlated with disease production. Moreover, the constancy of these fermentation characters in streptococci has been called in question.

Agglutination by antiserum has been another method very frequently attempted for such a division. Early workers—Aronson (1903), Neufeld (1903)—found agglutination heterologous within the hæmolytic group, and Besredka (1904) agreed with their results. Some years later Floyd and Wolbach (1913) noted some evidence of such a grouping, but Kligler (1915) found no clear-cut division by agglutination methods. However, more recently, Avery, Dochez and Lancefield (1919), Havens (1919), Tunnick (1920) and Bliss (1920) have obtained much more encouraging results.

## SCOPE OF PRESENT RESEARCH.

This work was undertaken in an attempt to obtain evidence for or against the possibility of classifying streptococci by means of agglutination. From the beginning numerous hæmolytic strains gave difficulty because of spontaneous agglutination, and as time went on stable suspensions of non-hæmolytic strains became increasingly more difficult to obtain. Various media, solid and fluid, with varying pH and serum-content were tried, in the hope of finding conditions which would consistently be favourable to a growth which could be used for agglutination purposes. The results, however, were so variable under apparently similar conditions, that it seemed there must be something variable in the organisms themselves, which was not altogether a function of their surroundings.

## DEMONSTRATION OF DISSOCIATION INTO "ROUGH" AND "SMOOTH" TYPES.

The possibility of the presence of "rough" and "smooth" types in streptococcal cultures similar in properties to those found by Arkwright (1921) in some members of the intestinal group of bacteria presented itself. The "smooth" types of *B. typhosus* and *B. paratyphosus* grow in "regular" colonies, easily emulsifiable and not liable to spontaneous agglutination, whereas the "rough" types grow in "irregular" colonies, not easily emulsifiable and tending to agglutinate in normal saline.

Broth cultures of several strains of streptococci, 8 hæmolytic and 2 non-hæmolytic, were plated. From the plates numerous colonies were picked to serum broth and incubated for twenty-four hours. From the tube showing the most even turbidity, and that with least, plates were again made. The most even and the most irregular colonies were picked to serum broth. These operations were repeated over a period of about four months before a definite difference could be relied upon to appear in every culture. But each strain worked with has finally yielded two definite types of organisms—"roughs" and "smooths."

*Characters of "Rough" and "Smooth" Types.*

The "smooths" grow with even turbidity in broth, and form bluish translucent colonies with even outline and a very finely granular surface on agar. The "roughs" grow as a precipitate in broth, and on agar have white, more opaque, coarsely granular colonies with irregular outlines, often appearing as a tangled mass of one long chain of organisms. Microscopically the "roughs" show more clumps and much longer chains than the "smooths"; also *more variation in size* with a distinct tendency to be larger. Some of the "rough" forms also have a mucous growth in broth. No capsules have been demonstrated.

*Hæmolysin Production.*

The power of the two types to produce hæmolysin was tested on sheep's blood-corpuscles, the broth cultures being centrifuged to prevent any action on the part of the organisms themselves, so far as possible. First results seemed to indicate that the "smooth" type was much more active in producing



hæmolysin than the "rough" type. However, it was observed that in a mucoid "rough" culture, when some strands of growth were suspended in the fluid, there was a greater amount of hæmolysin than if the growth were all in the bottom of the tube. The probability was that the active hæmolytic substance was closely allied to the organisms, and did not readily diffuse from a sediment growth as was found by Lyall (1914). To ascertain whether this was the case eighteen-hour cultures of "roughs" were tested to see whether a difference existed between the hæmolytic activity of the fluid carefully pipetted from the top of the cultures and of the fluid immediately surrounding the organisms. The difference was very marked, the upper layer giving no hæmolysis or partial in low dilutions while the lower layer gave complete hæmolysis in varying dilutions.

The action of "roughs" and "smooths" on blood plates was also tested. Five strains were plated on 10 per cent. rabbit's blood agar. There was some variation in the size and completeness of the ring of hæmolysis around the colonies, but this depended on the size of the colony and not on whether it was "rough" or "smooth." The results were the same for three tests of the series.

Since obtaining these results all cultures to be tested for hæmolytic activity have been well shaken before centrifuging, and "roughs" and "smooths" have then been found to agree very closely in hæmolytic property.

TABLE I.—*Hæmolytic Activity.*

Strains.	Dilutions of broth cultures with degree of hæmolysis.				
	1:2.	1:4.	1:8.	1:20.	1:40.
First results:					
"H. Cohen," rough . . .	—	—	—	—	—
smooth . . .	C	C	C	AC	AC—
"2384," rough . . .	AC	AC+	Tr	—	—
smooth . . .	C	C	C	C	C
"Crowe," rough . . .	C	AC	AC—	Tr	—
smooth . . .	C	C	C	C	C
Top and lower layer of rough cultures:					
"H. Cohen," R., top layer . . .	C	C	AC	Tr+	Tr
R., lower layer . . .	C	C	C	C	C
"2384," R., top layer . . .	C	C	AC	Tr+	Tr
R., lower layer . . .	C	C	C	C	C
"Crowe," R., top layer . . .	—	—	—	—	—
R., lower layer . . .	C	C	C	Tr+	Tr+
Estimations after shaking:					
"H. Cohen," rough . . .	C	C	C	C	C
smooth . . .	C	C	C	C	C
"2384," rough . . .	C	C	C	C	Tr+
smooth . . .	C	C	C	C	C
"Crowe," rough . . .	C	C	C	Tr+	Tr
smooth . . .	C	C	C	Tr+	Tr
C = Complete hæmolysis.			Tr	= Trace of hæmolysis.	
AC = Almost complete hæmolysis.			Tr+	= More than trace of hæmolysis.	
AC— = Less than almost complete hæmolysis.			—	= No hæmolysis.	

*Virulence.*

*Preliminary observations.*—The virulence of the two types of the 8 hæmolytic strains was tested on mice and rabbits.

A corresponding number of untreated mice were inoculated as controls. Little immunity resulted, as at the end of 3 days only 1 mouse previously treated with "rough" was living. However, this may have been due to the fact that the dose was considerably above the minimal lethal dose.



*Experiment II.*—The “rough” and “smooth” types of 1 strain were given to mice (in duplicate) in quantities of 1 slope  $\frac{1}{2}$ ,  $\frac{1}{4}$ ,  $\frac{1}{8}$ ,  $\frac{1}{16}$ ,  $\frac{1}{32}$  slope. Thus in all 12 received “rough” and 12 received “smooth.”

In 3 days 7 inoculated with “smooth” were dead but none inoculated with “rough.” The remaining mice were kept under observation for 10 days to give them the chance to develop protective bodies. At the end of 10 days only 10 of the 17 survived, viz. 10 of the original 12 mice inoculated with “roughs.” These 10 were now given 0.5 c.c. of a “smooth” culture of the same strain. One control was also injected with this quantity which had previously been found to kill 4 out of 6 mice in 24 hours. The results were as follows: Two reinjected mice died overnight; control mouse died in 48 hours; 1 reinjected mouse died in 3 days.

Thus 7 out of 10 mice which had survived inoculation with “roughs” had undoubtedly acquired a very considerable degree of immunity to the corresponding “smooth” type.

*Examination of peritoneal exudate after inoculation.*—To discover, if possible, whether any difference could be detected in the behaviour of “roughs” and “smooths” after injection into the peritoneal cavity of mice, one mouse was given 0.5 c.c. of a 24-hour broth culture of “rough” and one the same amount of “smooth” of the same strain. Fluid was removed from the peritoneal cavity after 30 minutes, 1 hour, and then hourly up to 8 hours. Four strains were investigated in this way.

During the first two hours the “smooths” showed slight decrease or no change in numbers. A rapid increase then followed and 3 of the 4 mice died overnight. The remaining mouse recovered, and it was observed that in the case of this animal the organisms decreased from the fourth hour onwards. With regard to the “roughs” only a few cocci were present after 30 minutes. In one case the cocci remained fairly constant until the second hour, when a rapid decrease ensued.

In all cases lymphocytes and a very few polymorphonuclear leucocytes were found during the first hour; from the second hour the number of polymorphonuclear leucocytes increased and this increase continued during the period of examination. With the “smooths” the increase was much less than with the “roughs.” Some phagocytosis was seen but never to any marked degree.

*Late examination of mice which had survived intraperitoneal inoculation of “rough” types.*—Mice injected intraperitoneally with “rough” types and which died or were killed four or six days or even weeks later showed abscesses in the abdominal cavity. These varied in size from that of a small pea to one including almost one whole lobe of the liver. The greatest number have been in the splenic omentum. Some occurred also on the surface of the liver, such sites suggesting almost certainly a purely local lymphatic spread from the peritoneal cavity. The abscesses were walled off. From each abscess a “rough” growing hæmolytic streptococcus was isolated. In two mice which were killed 8–10 days after inoculation with “smooths,” small abscesses were also found in the peritoneal cavity, and from these cultures of hæmolytic streptococci which were a mixture of “rough” and “smooth” were grown. A mice which survived a “smooth” injection for six days and then died



showed no abscesses, but a hæmolytic "smooth" streptococcus was isolated from the heart's blood.

TABLE II.—*Virulence.*

Strain.	Dilution of culture giving complete hæmolysis.	Animals injected.	Dose.	Death or survival.
First series:				
"2384," rough .	1 : 20	2 mice	0.5 c.c. 24-hr. broth culture	Both alive on 7th day.
" " smooth	1 : 20	2 "	Do.	† 24 hrs. † 48 hrs.
"W.," rough .	1 : 8	2 "	"	† 24 hrs. Alive on 7th day.
" " smooth	1 : 8	2 "	"	† 24 hrs. † 48 hrs.
"L.," rough .	1 : 4	2 "	"	Both alive on 7th day.
" " smooth	1 : 4	2 "	"	Both † 24 hrs.
"B.C.," rough .	1 : 40	2 "	"	Both alive on 7th day.
" " smooth	1 : 40	2 "	"	Both † 24 hrs.
"Crowe," rough	1 : 8	2 "	"	Both alive on 7th day.
" " smooth	1 : 8	2 "	"	Both † 24 hrs.
"2716," rough .	1 : 8	2 "	"	Both alive on 7th day.
" " smooth	1 : 8	2 "	"	Both † 24 hrs.
"A.B.," rough .	1 : 8	2 "	"	Both alive on 7th day.
" " smooth	1 : 8	2 "	"	"
"H. Cohen," rough .	1 : 20	2 "	"	† 24 hrs. Alive on 7th day.
" " smooth.	1 : 20	2 "	"	Both † 24 hrs.
Second series:				
"2384," rough .	1 : 20	2 "	"	† 6 days. Alive on 7th day.
" " smooth	1 : 40	2 "	"	Both † 24 hrs.
"W.," rough .	1 : 40	2 "	"	† 24 hrs. and † 6 days.
" " smooth	1 : 40	2 "	"	Both † 24 hrs.
"L.," rough .	1 : 8	2 "	"	Both alive on 7th day.
" " smooth	1 : 8	2 "	"	Both † 24 hrs.
"B.C.," rough .	1 : 40	2 "	"	Both alive on 7th day.
" " smooth	1 : 40	2 "	"	Both † 24 hrs.
"Crowe," rough	1 : 20	2 "	"	† 48 hrs. and † 72 hrs.
" " smooth	1 : 20	2 "	"	Both † 24 hrs.
"2716," rough .	1 : 20	2 "	"	† 6 days. Alive on 7th day.
" " smooth	1 : 20	2 "	"	Both † 48 hrs.
"A.B.," rough .	1 : 20	2 "	"	† 48 hrs. Alive on 7th day.
" " smooth	1 : 20	2 "	"	Both alive on 7th day.
"H. Cohen," rough	1 : 40	2 "	"	Both † 24 hrs.
" " smooth.	1 : 40	2 "	"	"
"R.," rough .	non-hæm.	2 "	"	Both alive on 7th day.
" " smooth	"	2 "	"	"
"Infrequens," rough .	"	2 "	"	"
" " smooth	"	2 "	"	"
"2384," rough .	—	1 rabbit (900 grm.)	1-24-hr. slope	† 10 days. No apparent lesions.
" " smooth	—	1 rabbit (900 grm.)	"	† 6 days. No apparent lesions.
"W.," rough .	—	1 rabbit (1100 grm.)	"	Survived.
" " smooth	—	1 rabbit (1120 grm.)	"	† 24 days. No apparent lesions.
"L.," rough .	—	1 rabbit (800 grm.)	"	Survived.
" " smooth	—	1 rabbit (900 grm.)	"	† 6 days. Slight gelatinous exudate around heart.

TABLE II.—*Virulence*—continued.

Strain.	Dilution of culture giving complete hæmolysis.	Animals injected.	Dose.	Death or survival.
Second series—continued:				
"B.C.," rough . . .	—	1 rabbit (1200 grm.)	1-24-hr. slope .	Survived.
„ smooth . . .	—	1 rabbit (1400 grm.)	„	† 10 days. No apparent lesions.
"Crowe," rough . . .	—	1 rabbit (1000 grm.)	„	Survived.
„ smooth . . .	—	1 rabbit (1020 grm.)	„	† 8 days. No apparent lesions.
"2716," rough . . .	—	1 rabbit (1280 grm.)	„	Survived.
„ smooth . . .	—	1 rabbit (1350 grm.)	„	† 6 days. Small abscesses in liver and kidney.
"A.B.," rough . . .	—	1 rabbit (1150 grm.)	„	Survived.
„ smooth . . .	—	1 rabbit (1150 grm.)	„	Survived.
"H. Cohen," rough . . .	—	1 rabbit (1200 grm.)	„	Survived.
„ smooth . . .	—	1 rabbit (1220 grm.)	„	† 6 days. Small abscesses on kidneys.

† = Death. Mice inoculated intraperitoneally. Rabbits inoculated intravenously. .

## DISCUSSION.

The presence of "roughs" and "smooths" in streptococcal cultures would account for many of the difficulties encountered in agglutination work because of the fact that the "roughs" grow in clumps, and it remains to be determined now whether these types are distinguishable serologically. This question is being considered.

The mechanical or physical factor in the diffusion of the hæmolysin would account for decrease in the hæmolytic activity of a culture following an increased proliferation of the "rough" elements.

The most striking outcome, however, of these researches is the demonstration of an almost complete lack of virulence on the part of the "rough" elements in streptococcal cultures. Accurate knowledge has hitherto been wanting on this highly important association of virulence differences with colony form, but it is of much interest that de Kruif (1921) has recorded a very similar association in connection with a strain of *Pasteurella*.

So far as one may judge from the intra-peritoneal inoculations in mice, it would seem that the mechanical arrangement of the "roughs" in clumps might go far to explain the tardy and only very local proliferation in tucked-away corners of omental tissue, the consequent abeyance of blood invasion, and the final disposal of the infecting "roughs" in localised walled-off foci through the agency of the peritoneal lymphatics.

The clumps are in fact dealt with exactly like inorganic foreign particulate matter such as melanin when introduced into the peritoneal cavity (Ledingham, 1909). The ultimate fate of these localised foci is yet uncertain, but it is of

considerable interest that their presence is associated with a definite acquired immunity to the corresponding virulent "smooth" type.

While the sequence of events after intraperitoneal inoculation of "roughs" in the mouse took this particular form, there was no evidence that rabbits which survived *intravenous* inoculation with "roughs" harboured such foci when later submitted to autopsy. The mechanical factor, therefore, will only partially explain the lack of virulence or invasive power.

Finally, a consideration of the data relating to degree of hæmolytic activity and associated virulence (detailed in Table II) would indicate that hæmolysin-production has not the close relationship with virulence which many workers have alleged, and the results in this respect are in agreement with those of Lyall (1914), Longcope, Stevens and Brady (1920), and Stevens, Brady and West, (1921) whose work lends no support to any such intimate relationship.

#### SUMMARY.

(1) Individual strains of streptococci can be "dissociated" into "rough" and "smooth" types which show remarkable differences in virulence for laboratory animals.

(2) Inoculation with the "rough" or avirulent type appears to afford considerable protection against the "smooth" or highly virulent form.

(3) Hæmolysin production is not a dominating factor in streptococcal virulence.

I wish to thank Dr. Ledingham for much helpful advice and criticism in the course of this work.

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## EXPERIMENTS ON THE INFECTIVITY OF TYPHUS VIRUS CONTAINED IN LICE (*PEDICULUS HUMANUS* AND *PEDICINUS LONGICEPS*).

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THE experiments detailed in this paper were performed with two distinct strains of typhus virus. The first strain used, for which our thanks are due to the League of Red Cross Societies Typhus Research Committee, was brought from Warsaw by one of us in the summer of 1920, inoculated in guinea-pigs.

After a number of passages through guinea-pigs, a monkey and human lice, this virus died out, whether from attenuation or not it is difficult to say. Through the kindness of Prof. A. Stokes, of Dublin, we obtained some guinea-pigs inoculated with the blood of a typhus patient from Ireland.

The monkeys used were *Macacus rhesus* except one, which was a *Macacus* of another species (unidentified).

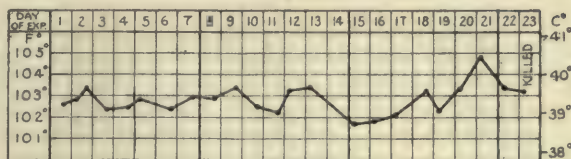
### THE INFECTION OF GUINEA-PIGS BY THE INJECTION OF INFECTED HUMAN LICE.

In common with previous workers we found no difficulty in infecting guinea-pigs with emulsions in salt solution of human lice which showed a heavy infection with *Rickettsia prowazeki*. The dose of virus, even when only one or two lice are used, is large in comparison with guinea-pig to guinea-pig injection, judging by the early reactions of temperature in the animal, in some cases as early as the fifth or sixth day.

Two methods of infecting the human lice were employed in the experiments detailed in this and other sections of the paper: (1) By feeding the insects on a typhus-infected monkey, and (2) by the rectal injection (Bacot, 1922) of virus obtained by centrifuging blood of typhus-infected guinea-pigs or the emulsified alimentary system of a previously infected louse.

*Experiment 1.*—The alimentary tracts of two living human lice which had fed on a typhus-infected monkey for 8–11 days were emulsified in salt solution and injected into the peritoneal cavity of guinea-pig No. 38.

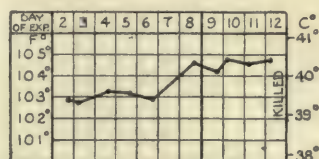
The heart blood of this guinea-pig infected, on inoculation, another, which showed typical brain lesions. The temperature reaction of this guinea-pig was exceptionally late (20th-21st day).



Temperature Chart, Guinea-pig No. 38.

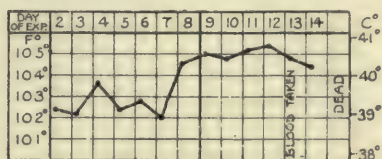
*Experiment 2.*—The alimentary tracts of 3 human lice which had fed on a typhus-infected monkey were emulsified in salt solution and injected into the peritoneal cavity of guinea-pig No. 44.

Sections of the brain showed typical lesions, and the blood and brain infected other guinea-pigs.



Temperature Chart, Guinea pig No. 44.

*Experiment 3.*—The alimentary tracts of 7 human lice, which had been infected by the injection, *per rectum*, of an emulsion of the alimentary system of another infected louse, were injected subcutaneously into guinea-pig No. 148. Six of these lice had died in an interval of two days preceding the injection, but the seventh survived. They constituted the fourth passage through lice from the original batch which had been injected with platelet material\* from the blood of a typhus-infected guinea-pig.



Temperature Chart, Guinea-pig No. 148.

#### FAILURE TO INFECT MONKEYS OR A GUINEA-PIG BY BITES OF INFECTED HUMAN LICE.

The infected lice were fed on an infected area of skin that had been shaved on the previous day. All possible care was taken to avoid contamination of the animal's skin with excreta from the lice, which were closely watched while feeding, and any faeces passed during the process were, if possible, removed before coming into contact with the skin. In the few instances where contact was unavoidable the excreta were removed at once, and the spot on which they had fallen was sponged over with a minute swab of cotton-wool soaked with 2 per cent lysol. Between the intervals of feeding the lice were kept in an incubator at a temperature of about 32° C. (90° F.).

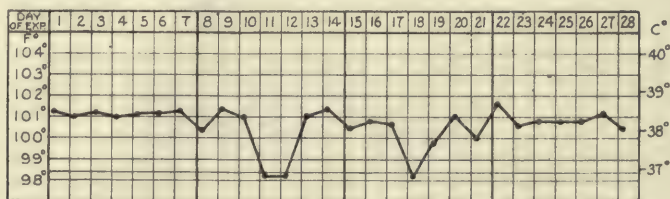
*Experiment 4.*—Six human lice infected by the rectal injection of platelet material on 30 . ix . 21 and ten that had been similarly infected on 5 . x . 21 were fed by the rectal injection of human blood until 7 . x . 21, when they were given one feed upon monkey No. 4, and subsequently twice daily for five days. During this period the insects were dying off rapidly and only two survivors

\* We are indebted for this material to Dr. Ségat.



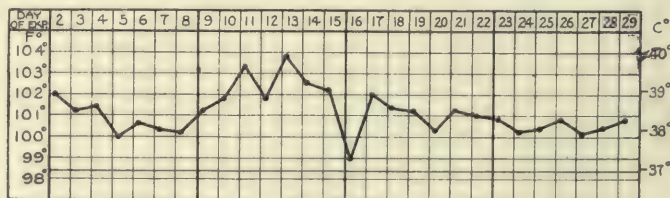
lived to feed on the fifth day. All the lice which died showed *Rickettsia prowazeki* on microscopic examination, and as the two survivors were too feeble to feed after the fifth day their emulsified alimentary tracts were inoculated into two guinea-pigs, both of which developed typhus.

Monkey No. 4, however, developed no fever.



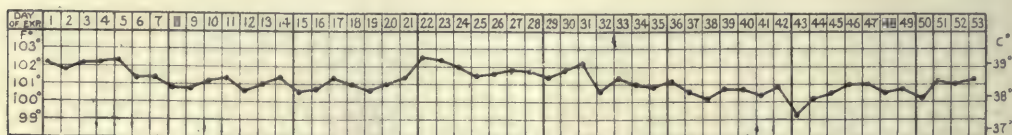
Temperature Chart, Monkey No. 4 (1st).

Half the brain of an infected guinea-pig was injected subcutaneously into this monkey a month later and produced a well-marked typical attack of fever in the second week, showing that the monkey was not immune and that the lice in all probability had failed to infect it.



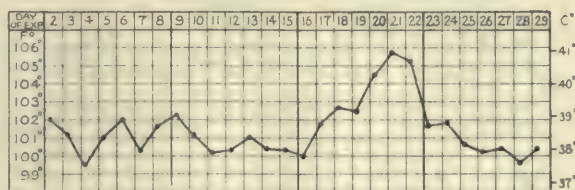
Temperature Chart, Monkey No. 4 (2nd).

*Experiment 5.*—Twenty-three human lice which had been infected by feeding on a typhus-infected monkey (No. 4) twice daily for a week, commencing at the period when its temperature was at its height, were fed twice daily on monkey No. 5. Their numbers, however, rapidly dwindled until there were only three living on the 5th and last day of feeding. Smears made from two of the three showed *Rickettsia prowazeki* in an early phase of development. There is little doubt that these lice contained the virus of the disease, yet the monkey showed no rise of temperature.



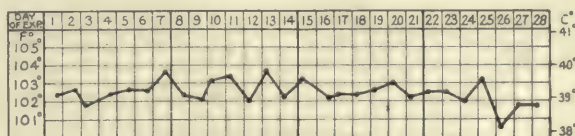
Temperature Chart, Monkey No. 5 (1st).

A test dose of virus, half a brain of an infected guinea-pig, was injected subcutaneously into this monkey about two months after the beginning of the experiment and produced a well-marked temperature reaction, proving that the animal was not immune.



Temperature Chart, Monkey No. 5 (2nd).

**Experiment 6.**—Fourteen human lice which had been infected by feeding on a typhus-infected monkey (No. 1) twice daily for 10–13 days were given one feed on guinea-pig No. 43.



Temperature Chart, Guinea-pig No. 43 (1st).

This guinea-pig was subsequently infected by the injection of a portion of brain of an infected guinea-pig, proving it not to be immune.



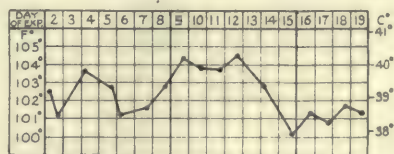
Temperature Chart, Guinea-pig No. 43 (2nd).

Of the 14 lice fed upon it 12 fed fully, one fed slightly, and one did not obtain any blood. Smears made from these lice subsequently showed that nearly all were infected with *Rickettsia prowazeki*, most of them heavily, and an emulsion of three of them produced typhus in guinea-pig No. 44 referred to above (Experiment 2).

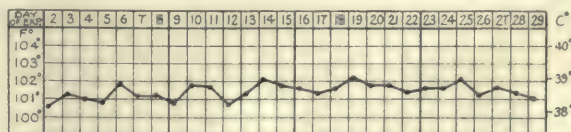
#### THE INFECTION OF MONKEYS AND ATTEMPTED INFECTION OF GUINEA-PIGS BY THE VIRUS CONTAINED IN INFECTED MONKEY LICE (*PEDICINUS LONGICEPS*).

**Experiment 7.**—The alimentary canals dissected out of 36 monkey lice were emulsified in salt solution and injected subcutaneously into the thigh of monkey No. 2. These monkey lice, with others, were captured on a typhus-infected monkey (No. 1) on the 12th day after its initial rise of temperature in response to the injection of infected guinea-pig brain. Smears made from the emulsion injected into monkey No. 2 as well as smears made from individual specimens of the captured lice showed *Rickettsia prowazeki*.

Eight months later this monkey received an injection of half a brain of an



Temperature Chart, Monkey No. 2 (1st).



Temperature Chart, Monkey No. 2 (2nd).

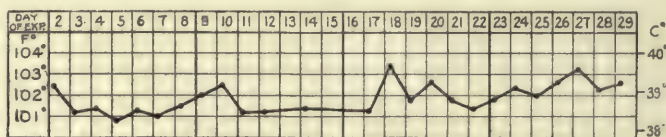


infected guinea-pig, but there was no rise of temperature, showing that it was immune.

*Experiment 8.*—Seven living monkey lice were transferred from monkey No. 2 to monkey No. 3 at a time when they might be expected to be infected with the typhus virus. This monkey's temperature was taken for 26 days but did not rise. The animal died on the 13th day after we had ceased to take its temperature. A large number of monkey lice were collected from the body, and a microscopic examination of smears made from 46 of these lice showed 31 of them to be infected with *Rickettsia prowazeki*.

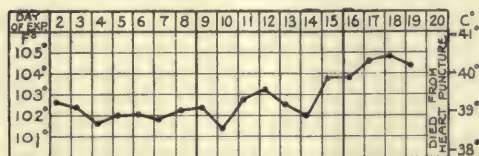
Arkwright, Bacot and Duncan (1919) have recorded that they examined normal monkey lice and found them free from *Rickettsia*. One of us (B.), when in Warsaw, examined a further series, and in the course of this work we have examined yet another series. In all, smears made from 150 *Pedicinus longiceps* taken from normal monkeys have been examined without any traces of *Rickettsia* bodies being discovered. We therefore assume on the strength of the presence of *Rickettsia prowazeki* in the lice taken from this monkey after its death that it developed an attack of typhus subsequent to our lapse in recording its temperature.

*Experiment 9.*—An emulsion of 100 monkey lice taken from monkey No. 3 (Experiment 8) was injected into the peritoneal cavity of guinea-pig No. 65, but no rise of temperature indicating typhus occurred.



Temperature Chart, Guinea-pig No. 65 (1st).

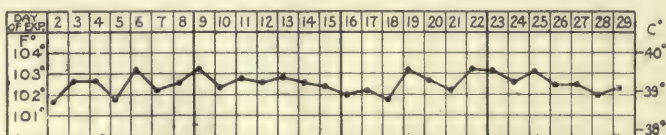
Ten months later a quarter of a typhus-infected guinea-pig brain injected into the peritoneal cavity produced typical fever, showing that the animal was not immune.



Temperature Chart, Guinea-pig No. 65 (2nd).

A smear of the emulsified guts showed the presence of *R. prowazeki*.

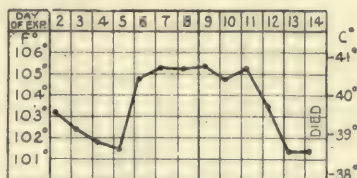
*Experiment 10.*—The alimentary tracts of five monkey lice, taken from monkey No. 4 (Experiment 4) about a fortnight after the height of its fever, were emulsified in salt solution and injected subcutaneously into guinea-pig No. 147 without producing any rise of temperature.



Temperature Chart, Guinea-pig No. 147 (1st).



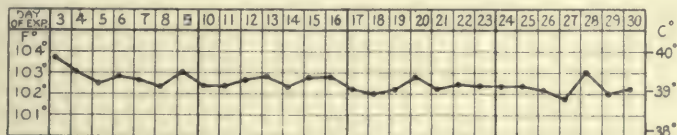
Seven weeks later a quarter of a typhus guinea-pig brain produced the usual well-marked rise of temperature.



Temperature Chart, Guinea-pig No. 147 (2nd).

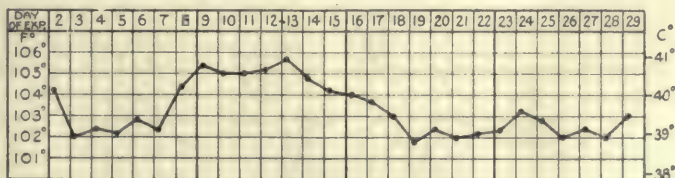
#### ATTEMPT TO INFECT A GUINEA-PIG WITH THE EXCRETA OF TYPHUS-INFECTED HUMAN LICE.

*Experiment 11.*—The excreta used for this experiment were collected on two consecutive days from the lice fed on monkey No. 4. They were collected as they were ejected by the insect with a fine sable-haired paint brush moistened with salt solution and were put in a small tube containing a dish of salt solution. Those collected at the evening feed were placed in the same tube as those collected in the morning, each day's collection being injected into the guinea-pig the evening of the day on which it had been collected. Three of the lice fed on monkey No. 4 (which probably contributed to the excreta used for inoculation) were emulsified and inoculated into three guinea-pigs. One died some days after the injection with a high temperature; the other two both showed typical typhus charts. The two injections of excreta produced no rise of temperature.



Temperature Chart, Guinea-pig No. 131 (1st).

Subsequently this guinea-pig received an injection of a quarter of a brain of a typhus-infected guinea-pig. Its temperature reaction in the chart below shows that it was not an immune animal.



Temperature Chart, Guinea-pig No. 131 (2nd).

#### COMMENTS.

As noted by previous workers, who have compared strains of typhus virus from various countries, we found that the Polish and Irish strains were apparently identical. Guinea-pigs which had survived attacks of typhus

caused by inoculation with the Polish strain proved to be immune to the Irish virus.

Although our experiments were not planned with a view to determining if *Rickettsia prowazeki* is the causal organism of typhus, all the evidence obtained bearing on this question tends to support the view that it is so.

It seems rather remarkable that while there was no difficulty in infecting monkeys with typhus through the medium of monkey lice (if the evidence in the case of monkey No. 3 in Experiment No. 8 be admitted, it was a case of natural transmission), yet all three attempts to transmit the disease to guinea-pigs by the injection of emulsified *Pedicinus longiceps* taken from the infected monkeys failed. Unless this failure be attributed to mere chance it would seem to suggest a lowered virulence in the virus when passed through monkey lice.

More definite is our failure in the case of both monkeys and a guinea-pig to infect by bite. Although the number of trials were few they appeared to be favourably circumstanced, except, of course, that the lice were not feeding on their natural host, but this, it will be remembered, has proved no bar to insect transmission by bite in the case of other diseases. The only other anomaly which is of some importance as a negative result was obtained, is that in the case of the monkey experiments the lice used were not infected in the natural way by feeding on an infected animal, but by rectal injection of typhus virus.

The evidence obtained from smears made from the salivary glands carefully dissected out of a few lice heavily infected with *Rickettsia prowazeki* tends to negative any suggestion of the transmission of typhus by the medium of the salivary fluid. In no instance were smears made of the large reniform glands, which are normally free from contact with the stomach infected. More suspicion attached to the forked vermiform salivary glands which lie on the top of the stomach, but the difficulty of avoiding contamination from the heavily infected cells of the stomach to the external surface of which the glands are attached by tracheal tubes, is very great. The most favourable method was found to be to cut off the fore end of the stomach after dissecting out the alimentary tract, remove it to a clean drop of salt solution, and gradually tease away fragments of the stomach from the attached glands; it was seldom found possible to obtain both glands free from injury. When freed from the stomach the glands were passed through clean salt solution and then teased. The numbers of *Rickettsia prowazeki* observed in these smears were few, if any, and in any case were infinitesimal compared with the numbers present in the smear of the drop of salt solution in which the operation of separating the glands from the fragment of stomach took place. The obvious conclusion was that the vermiform salivary glands were not infected, the number of *R. prowazeki* observed being more reasonably accounted for on the supposition that they were carried over from the heavily infected stomach-wall.

#### RÉSUMÉ.

(1) A Polish and an Irish strain of typhus virus appeared to be identical, as shown by cross-immunity experiments.

(2) Guinea-pigs were infected by the injection of the emulsified alimentary tracts of human lice, which showed heavy infection with *Rickettsia prowazeki*. Some of these lice had been infected by feeding on typhus-infected monkeys, others by the rectal injection of platelet material obtained by the centrifugalisation of blood from infected guinea-pigs.

(3) One monkey was infected with typhus by subcutaneous inoculations of infected monkey lice (*Pedicinus longiceps*), and a second was probably infected by the transference of living monkey lice from an infected monkey.

(4) Two attempts to infect guinea-pigs by the injection of infected monkey lice failed.

(5) Attempts to infect monkeys and a guinea-pig by feeding infected human lice upon them failed.

(6) An attempt to infect a guinea-pig with the excreta taken from heavily infected human lice failed.

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## THE RELATION OF NATURAL DIPHTHERIA ANTITOXIN IN THE BLOOD OF MAN TO PREVIOUS INFECTION WITH DIPHTHERIA BACILLI.

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It has always seemed likely to some that natural diphtheria antitoxin appears in man as a result of previous infection with diphtheria bacilli. However, it has been very hard to prove this hypothesis. The Schick test has rather added to the difficulty, because the number of individuals who are Schick negative is too great to be accounted for by previous attacks of diphtheria. And again, it is observed in many cases that an actual attack of diphtheria produces no natural antitoxin subsequently. The suggestion may be made that the antitoxin might be due to previous "carrier" infection, but this explanation also becomes unsatisfactory in view of the universal experience of all workers that virulent "carriers" have negative Schick reactions, *i. e.*, are already immune, the Schick positive healthy "carrier" being unknown.

Owing to a fortunate combination of circumstances, some experimental Schick tests carried out at a residential school seem capable of throwing light on the subject, and supporting the reasonable view that immunity to diphtheria is caused by recognised or unrecognised contact with diphtheria bacilli.

### MATERIAL AND TECHNIQUE.

The school consists of 950 boys ranging from 11 to 16 years of age. They are all as physically perfect as possible, being selected by a strict medical examination with a view to ultimate service in the Royal Navy. It should be noted, in particular, that special care is taken of the teeth, noses and throats of these boys. On the whole they are of a social grade higher than the ordinary Council schoolboy, and therefore, according to most observers, more susceptible to diphtheria—that is, they should contain among them a higher proportion of Schick positive reactors. Diphtheria has been epidemic in the school from May, 1921, to the present time. The first series of Schick reactions were all performed in February and March, 1922, and my thanks are due to Dr. A. T. MacConkey and Dr. G. F. Petrie of the Lister Institute, who supplied and re-tested the necessary undiluted toxin. The same batch of toxin was used throughout, and when tested at the end of the investigation the m.l.d.

remained the same. The usual technique was employed; one-fiftieth of a m.l.d. in 0.2 c.c. of saline *without phenol* was the dose used throughout. A control injection was always made on the other arm with the same diluted toxin heated. After each batch of tests a control test was performed on a known Schick positive reactor, always using a much smaller dose than that used on the tested schoolboys. The result was always a typical positive reaction.

#### SCHICK IMMUNITY AND DIPHTHERIA PREVALENCE.

In the first series of tests 831 boys were examined. They can be divided into two groups: "new boys" who had not mixed with those already in the school, and "old boys" already in the school.

Diagram A, Fig. 1, shows the proportion of Schick positive reactors in these two groups. Nineteen new boys who joined in May, 1922, have been added to the original group of 67 "new boys" (Fig. 2) in order to increase the size of the sample. It is seen that 45 per cent. of 86 new boys were Schick positive reactors as against 14 per cent. of the boys already in the school—that is to say, the former contained proportionately three times as many boys susceptible to diphtheria as the latter. This difference is too great to be accounted for by the smallness of the sample of "new boys" or the difference in age. As regards age, among 19 new boys, aged 13 to 14 years, 8 (or 42 per cent.) were Schick positive reactors as compared with 14 per cent. of the same age among the "old boys." This difference in the percentage of non-immunes before and after joining the school at once suggested that the degree of immunity might be proportionate to the length of residence in the school.

It was possible by searching the school register to arrange the 831 Schick-tested boys in the order in which they had joined the school. A graph (Fig. 2) was then made as follows: The boys were divided into the batches who joined each term from September, 1917, when the most senior boys had joined the establishment. The fourteen terms since that date are written along the top of the graph; the number of boys joining at each of these periods is indicated by the figures along the bottom of the graph, and the graph itself is made by plotting out the percentage of Schick negative reactors among each

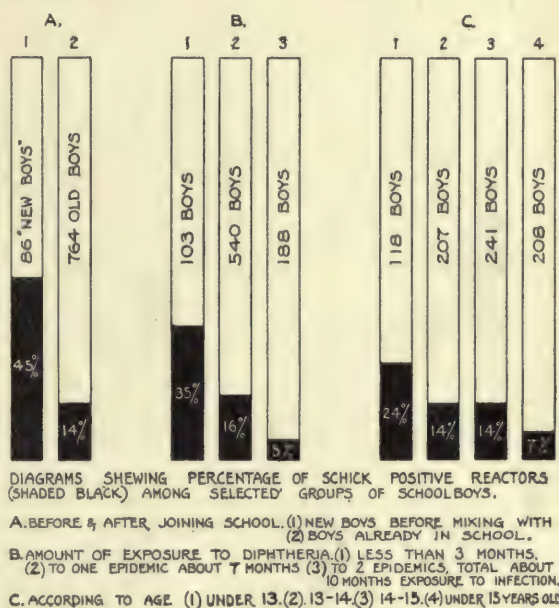


FIG. 1.



of the 14 samples of boys. The continuous line is a smoothed curve of the 14 points of reference thus obtained. This curve shows that the boys entering the school were under 60 per cent. immune according to their Schick reactions;

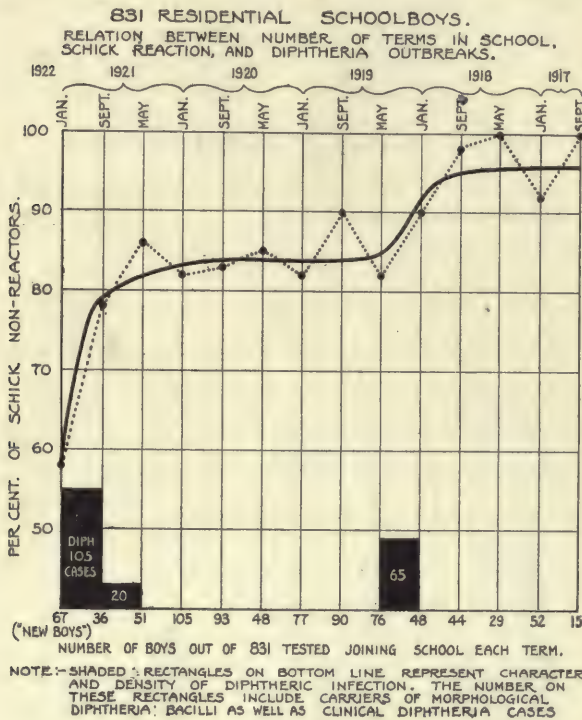


FIG 2.

and density of diphtheria infection as represented by the number of boys in whom bacilli morphologically resembling diphtheria bacilli were recorded as found. The school records show that there were 65 cases of diphtheria infection in the January to May term of 1919, after which, with the exception of an odd sporadic case or two, there is no entry until the start of the present outbreak, which commenced in May, 1921, reached its maximum in November, 1921, and has continued up to the present time. The diphtheria outbreaks are seen to coincide exactly with the steps in the graph, showing that immunising influences were working most rapidly in the presence of diphtheria. However, perhaps the most interesting feature is the horizontal line connecting the two steps, since it suggests that no immunisation of boys was taking place during the two years in which diphtheria was absent, because the sample of boys who entered the school in May, 1919, contains no more immunes than those who joined two years later in May, 1921, while those who joined before May, 1919, though only a few months longer in the school, contain 10 per cent. more Schick negative reactors. These senior batches had experienced an extra term of exposure to diphtheria infection.

Diagram B (Fig. 1) makes the relation between the schoolboys' Schick

boys with anything between six months' and two years' residence were 85 per cent. immune, while the oldest inhabitants, who had been  $2\frac{1}{2}$  to  $4\frac{1}{2}$  years at the school, were 95 per cent. immune to diphtheria. Now these alterations in degree of immunity occur by sudden steps, and not gradually, as they should if the immunising influence had been working throughout the whole period, and the degree of immunity was proportionate to the length of residence or age of the schoolboys. The shape of this curve—two steps connected by a horizontal line—is inexplicable until the diphtheria prevalence during the period under consideration is superimposed on the chart. The black rectangles on the bottom line indicate the duration



reactions and diphtheria prevalence plainer. In the first column the black portion is proportionate to those boys who had been exposed less than three months to diphtheria; they contained 35 per cent. Schick positive reactors. The second column represents the boys who had passed through seven months of the present diphtheria epidemic; they were 16 per cent. Schick susceptible, whereas the most senior group who had been through both epidemics, corresponding to a total of ten months' exposure to the risk of diphtheria infection, only contained 5 per cent. of non-immune boys. These diagrams do not, however, indicate the most important point, namely that no immunisation occurred in the absence of diphtheria, as is shown by the horizontal line in the graph.

Diagram C (Fig. 1) represents the 764 "old boys" arranged in four age-groups. The boys enter the school at from 11 to 14 years old but never remain after they are 16. If the appearance of natural antitoxin is a function of age in itself as distinct from a greater chance of infection, this diagram would show a regular series of steps from the youngest to the oldest boys. This, however, is not the case, as the two middle groups show no difference in immunity in spite of each of the samples exceeding 200. This diagram is merely the graph turned upside down. The youngest and oldest groups contain most of the junior and senior boys respectively, while the two middle groups comprise most of the boys who entered the school during the inter-epidemic period. Thus the age diagram still further confirms the deductions from the graph.

Assuming it is legitimate to apply the above figures generally, they mean that 45 out of every 100 boys who enter the school are Schick positive reactors, but on exposure to ten months of a diphtherial environment, such as existed in this school, 40 will become Schick negative reactors, in most cases without getting clinical signs of diphtheria—that is, 90 per cent. of positive reactors become negative reactors in ten months. Therefore a number somewhere in the neighbourhood of 27 per cent. should be found to change after three months' exposure to the same average concentration of diphtherial infection. To see if this reasoning was correct 100 unselected Schick positive reactors were re-tested after twelve to fourteen weeks had elapsed since their first tests. On the re-test 32 of these boys were found to have become negative reactors. Twenty-eight of these boys, who within three months had developed sufficient natural antitoxin to inhibit a Schick response, had no known history of diphtheria, but diphtheria cases and carriers were being recorded in the school all through the period between the two sets of tests. As it was very important that there should be no doubt that this result was not due to deterioration of the Schick toxin used, at the end of the re-test an injection of a two-thousandth of a m.l.d. (*i. e.* one-fortieth the Schick dose) was made into a known positive reactor with double the usual dose of heated toxin as a control, the same test solutions as those used on the re-tested boys being used. The usual typical positive reaction resulted, without any effect at the site of the control puncture. Another sample of boys consisting of 73 negative and 14 positive reactors were also re-tested thirty-four days after their first tests. None of the negative reactors had positive reactions, but two of the positive reactors had developed antitoxin within the month, becoming Schick negative. Thus the change in

these re-tests was always in the same direction—from positive to negative—never from immune to non-immune. In this way direct experiment proved that in three months 32 per cent. positive reactors had become negative in the presence of diphtheria, which approaches nearly to the 27 per cent. deduced from the graph (Fig. 2) drawn before the re-test was contemplated.

Recognised infection, that is, clinical diphtheria, according to the figures in this school, confers almost certain immunity. It is known that all diphtheria cases are Schick positive before attack. In 50 cases of clinical membranous diphtheria 42 were Schick negative and 8 positive reactors in from 3 to 6 months after therapeutic antitoxin had been given. Seven of the positive reactors were included in the re-tests mentioned above, 4 had become Schick immune, so that within nine months 92 per cent. of the post-diphtheritic cases had become Schick negative. Many observations in the literature show that a large proportion of post-diphtheritics have positive Schick reactions. Perhaps the high percentage which became Schick negative in this school is due to the fact that the convalescent diphtheria cases were returned into a diphtheria-saturated environment, where, if re-infected, they at once manufactured antitoxin instead of getting a second attack. This supposition is confirmed by the observation that no boy in this school had more than one attack of clinical diphtheria.

The missing link in the chain of evidence that natural antitoxin is due to infection with diphtheria bacilli is the Schick positive carrier of virulent bacilli. Over 200 throat cultures from healthy boys, who had been positive reactors at the time of the first series of tests, were examined. From 5 boys Neisser positive diphtheroids giving the fermentation reactions of *B. diphtheriae* were obtained, but only one culture was toxigenic. It is a curious coincidence that 3 of these 5 carriers were found to have become Schick negative reactors at the re-test, including the one virulent carrier. This latter, whose Schick test was positive ten weeks previously but negative a fortnight subsequent to the discovery of the virulent bacillus, is the nearest approach to a true Schick positive carrier found in this school. One of the other two avirulent Schick positive carriers developed diphtheria subsequently with virulent bacilli in his throat similar in all other respects to the harmless organisms he had been known to carry for over two months previously, which shows that in this case, at any rate, avirulent diphtheria bacilli had had no influence in producing immunity.

During the period of this investigation the carrier-rate for "morphological" diphtheria bacilli, as estimated from fair (743) samples of the school population, was 3.5 per cent. But the virulent carrier-rate as calculated from the bacilli that were isolated was nearer 1 per cent. than 2 per cent. This seems to be a low figure, but in this population the chronic carrier was unknown, and the virulent, as distinct from the avirulent carrier, very rarely remained infectious over a second weekly examination. The very transitory nature of the virulent carrier state in this establishment suggests that a large proportion of boys became infected with a very low average rate. If we allow the average duration of carrying to have been ten days and the rate to have been 1 per cent., it would have been possible for 20 per cent. of the boys to have been infected as *recognisable* carriers during the seven months of the



diphtheria epidemic preceding the Schick examinations, and if we include the actual cases of diphtheria the figure would be about 30 per cent., which is more than sufficient to give every boy in the school the chance of coming into contact with diphtheria bacilli, especially when it is realised how closely together these boys sleep. There are over 100 boys to a dormitory, closely packed into six rows of beds.

#### CONCLUSIONS.

In the school examined :

(1) "New" boys were three times as often susceptible to diphtheria as "old" boys.

(2) A graph is shown which indicates that the "old" boys became immune during outbreaks of diphtheria, and that between outbreaks no immunity was developed.

(3) The fact that the "old" boys were older was insufficient to account for the more frequent immunity.

(4) In the course of three months 32 per cent. of the susceptible boys became immune.

(5) In the course of nine months 92 per cent. of the boys who developed clinical diphtheria became immune.

(6) The period of "carrying" diphtheria bacilli was always short, and it is estimated that during seven months 30 per cent. of the boys were recognisable carriers.

(7) Under these circumstances it is probable that all boys in the school had the opportunity of being affected by the diphtheria bacillus to a slight unrecognisable degree.

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I wish to express my thanks to Surgeon-Captain P. M. May, R.N., and Surgeon-Commander S. Roach, R.N., for allowing me access to the school sick books. To the latter officer thanks are also due for his untiring work in arranging administrative details. Also acknowledgments are due to Surgeon-Lieutenant-Commander J. L. Priston, R.N., who helped with the Schick testing, and isolated many diphtheroids from the cultures I handed him.



## THE NATURE OF THE ACTION OF POTATO UPON THE GROWTH OF *B. INFLUENZÆ*.

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IN a previous paper (Fildes, 1921) it was argued that the action of blood pigment in the growth of *B. influenza* was similar to its action upon guaiaconic acid in the presence of a peroxide. This argument was based upon the parallelism, among other things, which was shown to exist between the action of the pigments upon guaiaconic acid and upon the growth of the bacillus. It was concluded that the pigment operated as a "peroxidase" or catalyst in connection with the supply of oxygen to the bacillus.

Thjötta and Avery (1921),<sup>2</sup> testing the effect of other substances which contain peroxidases, have shown that *B. influenza* will grow satisfactorily in ordinary broth containing fragments of sterile potato. This result, according to these authors, is due to the occurrence in the potato of two factors similar to those which they and others have described in blood. One they name the "X" factor, which corresponds to the blood pigment, and the other the "V" factor, which is comparable to the non-pigmented fraction of blood-corpuscles (Fildes, 1921). They demonstrated that the reaction of these two factors in potato to heat is similar to the reaction of the comparable factors in blood, namely the "X" factor will withstand 120° C. for 45 minutes, while the "V" factor will only survive 100° C.

This resistance to heat of the potato X factor makes it difficult to suggest that the stimulating action of potato is due to a peroxidase operating in the same manner as the blood-pigment "peroxidase," because the vegetable peroxidases are stated to be "inactivated by heating in solution for a short time" (Falk, McGuire and Blount, 1919). Further, Thjötta and Avery observed that banana contained no peroxidase, but would grow *B. influenza*. They concluded that the "identity of the X factor must remain a matter of more or less conjecture. In blood this substance seems to be associated with the iron-containing pigment."

The work of Thjötta and Avery discounts the suggestion that a peroxidase is a dominant factor in the growth of *B. influenza*, and experiments were therefore carried out to check it.

## PARALLELISM BETWEEN THE HEAT-STABILITY OF POTATO X FACTOR AND POTATO PEROXIDASE.

The statement that banana contains no peroxidase was not confirmed, but the progressive growth in broth containing sterile potato was readily obtained. Their technique, however, of using a fragment of potato in a tube of broth did not appear well adapted for judging the effect of heat, and so a potato juice was prepared to replace the more complicated whole vegetable. Peeled potatoes were passed through a mincing machine and then through a press. In this way a brownish fluid heavily charged with starch grains and *débris* was obtained. This was diluted with an equal quantity of saline solution and filtered through paper pulp, a coarse "English" Berkefeld candle, and finally a Berkefeld N to sterilise.

*Tests carried out in a slightly Alkaline Medium.*

The action of this potato juice, heated and unheated, upon the growth of *B. influenza* was then tested as follows:

*Experiment 1.*—Potato = fresh juice as above. Hæmatin = 2 per cent. solution, in a minimum of NaOH, of hæmin crystals separated from washed sheep's corpuscles by peptic digestion and washing; autoclaved. Yeast = watery extract of yeast according to Thjötta and Avery (1921).<sup>1</sup> Each tube contained 10 c.c. bactopectone water; broth was not used because it contains small quantities of Thjötta and Avery's V factor. The reaction of the peptone water was pH 7·6–7·9, and it did not fall below pH 7·6 upon the addition of the slightly acid yeast and potato. The additions were made with calibrated dropping pipettes, thus: potato 1 c.c., yeast 0·1 c.c., hæmatin 0·025 c.c. The tubes containing potato were heated by immersion in boiling water for 20 minutes or autoclaving at 120° C. for the same time. All inoculations were made by adding 0·05 c.c. of an extremely thin suspension of *B. influenza* from a tube containing a minimum of blood, in saline solution, the strength being about 40,000 viable bacilli per c.c. The amount of growth was judged by opacity checked by microscopic examination and subculture with a standard loop holding 0·003 c.c. to prove purity. The opacity of the tubes containing heated potato was difficult to judge owing to the presence of a coagulum, and therefore a doubt is indicated in the protocols.

The strain of *B. influenza* used in this work was No. 291 of the *National Collection of Type Cultures*, London.

	After 24 hours' incubation.		
	Opacity.	Microscopic.	Subculture. No. of colonies.
Bactopectone water :			
+ Hæmatin . . . . .	0	—	0
+ Yeast . . . . .	0	—	0
+ Hæmatin + yeast . . . . .	+	—	∞
+ Potato, unheated . . . . .	++	—	∞
+ do. + hæmatin . . . . .	++	—	∞
+ do. + yeast . . . . .	++	—	∞
+ Potato, heated 100° C. . . . .	0?	0	0
+ do. + hæmatin . . . . .	++?	very numerous	∞
+ do. + yeast . . . . .	0?	0	0
+ Potato, heated 120° C. . . . .	0?	0	0
+ do. + hæmatin . . . . .	0?	0	0
+ do. + yeast . . . . .	0?	0	0



From this experiment it is clear that the X factor of potato is destroyed not only by heating to 120° C. but also to 100° C.—a result which is in marked contrast to that of Thjötta and Avery. On the other hand the V factor, as they state, survives the latter temperature.

To test the effect of heat upon the potato peroxidase the same materials were put through the same process together with the tubes destined for cultures. They were then cooled and tested by adding a freshly prepared solution of guaiaconic acid in alcohol and a few drops of ozonic ether. With unheated potato juice in peptone water a blue colour rapidly develops, but this is absent in both heated specimens. Thus there is, in fact, a parallelism between the X factor and the peroxidase in potato as close as between the same two factors in blood.

### *Tests carried out in a Slightly Acid Medium.*

On considering further Thjötta and Avery's experiments, which gave results entirely in opposition to those here described, it appeared that they tested the heat stability by placing fragments of potato in broth, and thus the effect of heat might vary owing to the reaction of the substance of the potato being different. The reaction of the peptone water was pH 7·6–9, while the reactions of 12 different potato juices varied between pH 6 and 6·8. The previous experiment was, therefore, repeated, with the difference that the potato juice was heated in the slightly acid state *before* mixing with the peptone water.

*Experiment 2.*—Details as before. The first 6 tubes were as in Experiment 1 and are omitted.

	After 24 hours' incubation.		
	Opacity.	Microscopic.	Subculture.
Bactopeptone water :			
+ Potato, heated 100° C. . . . .	?	numerous .	∞
+ do. + hæmatin . . . . .	? + +	very numerous	∞
+ do. + yeast . . . . .	?	numerous .	∞
+ Potato, heated 120° C. . . . .	?	scattered .	numerous
+ do. + hæmatin . . . . .	?	scattered .	numerous
+ do. + yeast . . . . .	?	scattered .	∞

In this experiment the X factor (as also the V factor) is shown to be much more resistant to heat as stated by Thjötta and Avery. It is probable from the results of the microscopic examination that at 120° a quantitative reduction in power has taken place, but this is far from complete.

Turning now to the parallel test of the peroxidase enzyme with guaiaconic acid, it is found that samples heated to 100° C. for 20 minutes in the slightly acid state are *not* deprived of peroxidase action. The colour development is slow but quite marked, particularly when the heated tubes are left standing overnight before testing. Under the same conditions a tube diluted with peptone water before heating shows no colour.

Specimens heated to 120° C. do not, however, show an obvious peroxidase reaction with guaiaconic acid, although, as previously stated, these stimulate the growth of *B. influenzae*.



Thus at 100° C. there is still a parallelism between the survival of the X factor and the peroxidase in an acid medium, but this is not demonstrable at 120° C. It is, however, quite possible that the guaiaconic test is more gross than the growth test and that the latter might still give a positive result after the former had failed.

#### PARALLELISM BETWEEN THE ABSORPTION OF POTATO X FACTOR AND POTATO PEROXIDASE.

In Experiment 2 it was noticed that the blue colour of the guaiaconic test was confined to the coagulum produced in the potato juice by heating. The following experiment was therefore carried out to show whether the coagulum differed from the supernatant fluid in content of X factor.

*Experiment 3.*—Details as before. The samples of potato juice were heated in duplicate, and one of each pair was then centrifuged to supply the supernatant. The first 6 tubes were as in Experiment 1 and are omitted.

	After 24 hours' incubation.		
	Opacity.	Microscopic.	Subculture.
Bactotryptone water :			
+ Potato heated 100° C. supernatant .	0	—	0
+ do. + hæmatin . . . . .	++	—	∞
+ do. + yeast . . . . .	0	—	0
+ Potato, heated 100° C. deposit .	?	numerous	∞
+ do. + hæmatin . . . . .	?	„	∞
+ do. + yeast . . . . .	?	„	∞
+ Potato, heated 120° C. supernatant	0	—	1
+ do. + hæmatin . . . . .	0	—	100
+ do. + yeast . . . . .	0	—	0
+ Potato, heated 120° C. deposit .	?	isolated	very numerous
+ do. + hæmatin . . . . .	?	„	„
+ do. + yeast . . . . .	?	„	∞

From this experiment it is seen that the X factor is totally absent from the clear fluid after heating to 100° C., but is present in the coagulum. The same result is found after heating to 120° C. The guaiaconic test carried out on the same series shows that the peroxidase reacts in a similar manner. It is present in the deposit at 100° C., but not in the supernatant. As already stated, this test cannot be detected in the tubes heated to 120° C. It is probable that this absorption upon the coagulum has some bearing upon the resistance of the X factor and the peroxidase to heat (*cf.* Bayliss, 1918).

#### DISCUSSION.

The demonstration of the effect of potato upon the growth of *B. influenza* appears to throw light upon the view previously advanced as to the nature of the effect of blood pigment. It is shown that a peroxidase-containing substance, namely the potato, can take the place of blood-pigment, although the essential properties in potato and blood-pigment are quite different.

The conspicuous point of similarity between the two is the possession of

the power to oxidise guaiaconic acid in the presence of a peroxide. In a previous paper it was shown that the peroxidase factor in blood ran parallel with the power of the blood to induce growth of the bacilli, and in this paper it is demonstrated that the totally different peroxidase in potato has an almost identical reaction to heat and absorption as has the growth-inducing property of potato.

It is therefore suggested that the growth-inducing property (Thjötta and Avery's X factor) is associated with the peroxidase enzyme, its function being to accelerate the transfer of oxygen to the bacillus.

#### CONCLUSION.

It is suggested that the stimulating action of potato upon the growth of *B. influenza* is due to the action of the peroxidase enzyme operating in the same manner as blood-pigment to accelerate the transfer of atmospheric oxygen to the bacillus.

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## THE CULTIVATION OF *SPIRONEMA DUTTONI* FROM A CASE OF RELAPSING FEVER.

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THE spirochætes of relapsing fever were first cultivated by Noguchi (1912) from infected rats in his well-known tissue-ascites medium. He succeeded in propagating the different varieties for several generations. He found, however, that his cultures were viable only for a short time and that subcultures had to be made on the sixth or seventh day.

Starting with the Noguchi medium as basis, Kligler and Robertson (1922) developed a medium in which it was possible to cultivate consistently the spirochætes from rats, and in which the cultures remained viable for a period of three to seven weeks. The elements found to be essential for the growth and prolonged viability of these spirochætes were (a) a stabilised animal fluid having a reaction of pH 7·3, (b) a matrix of agar or fibrin, and (c) strict anaërobiosis. They observed that animal fluids (ascites, serum) become progressively alkaline on standing, and soon reach a reaction detrimental to the growth and life of the spirochætes, and that consequently the animal fluids used as substrates for the medium had to be neutralised and stabilised by means of some buffer.

On the basis of their observations these authors adopted the following medium: Horse or rabbit serum diluted 1 : 2 with saline, or undiluted ascitic fluid, is adjusted to pH 7·0 and 1·0 per cent. peptone added (1·0 c.c. of a 10 per cent. peptone solution to 10 c.c. substrate). The medium is distributed in 3 to 4 c.c. amounts into Wassermann tubes. Each tube is inoculated with 0·1 c.c. infected blood, mixed and covered with a layer of paraffin 1·5 cm. high. In initial cultures the inoculated blood furnishes the fibrin "basket"; in subcultures 0·1 c.c. of fresh rabbit blood is added for that purpose. The tubes are kept for 24 hours at 37° C. and then at room temperature (about 20° C.).

The only report to my knowledge of direct cultivation of the relapsing fever spirochætes from the blood of a patient is that by Plotz (1917), using the Noguchi technique. The object of the paper is to describe the direct cultivation of the spirochætes from the blood of a case of relapsing fever by the method developed by Kligler and Robertson.

*History of the case.*—Before giving the details of cultivation it will be of interest to review briefly the history of the case because of its atypical character.

M. W., male, aged 30 years, admitted to hospital 20/5/21 with intermittent temperature. The temperature on admission at 12 mid-day was 37·6° C. At 12 p.m. it rose to 38·9° C., fluctuated for four days and returned to normal.

After two days it rose again, remained up for three days, dropped for one day, and rose again for the third time the day after. The pulse was rapid during the first attack but remained normal thereafter. Blood drops and smears examined on the 20th, 21st and 23rd were negative. The blood-count was normal except for the high percentage of large mononuclears (8 per cent.). On the 26th at 9 p.m. spirochætes were found in the drops but another examination at 8 a.m. on the 27th was negative. At 12 mid-day 30/5/21 spirochætes were again found both in the thick drops and in the dark field. Cultures as well as rat inoculations were thereupon made with positive results. At 4 p.m. 0.45 grm. salvarsan was injected intravenously; the temperature dropped immediately and remained normal until dismissal (June 6th).

*Cultures of Sp. duttoni* (?).—Cultures were made on 30/5/21 in buffered ascites and serum media and also in bouillon. On 6/6/21 actively growing cultures were found in the ascites and serum media. Successful subcultures were made on 15/6/21 and 2/7/21. On 20/7/21 the cultures were lost through a mould contamination. Three generations of spirochætes were thus obtained with successful transplants after sixteen and eighteen days respectively.

The cultures were characterised by the absence of any change in the medium. The upper portion of the fibrin-blood "basket" remained bright red; the fluid portion was clear and tinged by a slight hæmolysis. Most of the spirochætes were found in the fibrin and only few in the clear fluid. In the fibrin masses one could see clumps (colonies?) as well as chains of two or three. If the culture remained clean and there was no marked hæmolysis, the spirochætes remained actively motile to the time of transfer. In some tubes degenerative changes characterised by rings on the spirochætes were observed. Any contamination promptly terminated the life of the spirochætes.

Rats inoculated with the second generation cultures, 2/7/21, became infected, and after forty-eight hours large numbers of spirochætes were found in the blood. The course of infection was the same as that produced with the patient's blood. Only one relapse was observed in the rats inoculated with culture material.

*Inoculation of rats.*—When the cultures were made, white rats were also inoculated intraperitoneally with the patient's blood. One to two days after the inoculation spirochætes appeared in the blood, increased in numbers for the next day or two and then disappeared, to reappear again after two days. No rat had more than one relapse. The strain was carried through three rat generations with the same results.

#### CONCLUSION.

It would seem that with the method described it is possible to cultivate the spirochæte of relapsing fever from the blood of patients and maintain it under artificial cultivation for long periods.

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## THE RELATIVE VOLUME OF CORPUSCLES AND PLASMA, AND THE RELATION OF THIS TO HÆMOGLOBIN PERCENTAGE AND THE NUMBER OF RED BLOOD-CORPUSCLES.

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WHILE using the hæmatocrit for some routine determinations of the relative volume of red cells in whole blood, it was obvious that this ratio depended less on the number of red cells present than on the hæmoglobin percentage.

The hæmatocrit was first used for this purpose by Heden (1891), and, more extensively, by Capps (1903), who introduced the term "volume index." This is obtained by dividing the relative volume of the corpuscles by the number of red cells (both being expressed as percentages of the normal), and gives a measure of the relative size of the red cell. Gram (1921) has recently published a somewhat similar investigation, 5 c.c. of blood being rotated in the centrifuge for ninety minutes. Biernacki (1894) allowed sedimentation of the corpuscles for twenty-four hours after adding potassium oxalate. Larrabee (1911), using this method, confirmed many of the results obtained by Capps.

### METHODS.

Venous blood was used for all experiments unless otherwise stated. Solid potassium oxalate sufficient to give a 0.5 per cent. solution was added.

In some experiments where blood was obtained in the usual way by pricking the finger and adding minimal quantities of potassium oxalate, it was much more difficult to get constant results, owing to the impossibility of getting any constant percentage of potassium oxalate (see appendix).

The hæmoglobin percentage was determined generally in duplicate by a specially standardised Haldane-Gowers hæmoglobinometer.

The red blood-corpuscles were counted with a Bürker-Zeiss hæmocytometer and at least two thousand were counted in each case.

The following method was used to determine the relative volume of corpuscles. Glass tubing was drawn out to capillary bore. Any part with obvious changes in diameter was thrown away, and the remaining length was cut up into capillary tubes of the required length. The blood was well mixed,



and six tubes were dipped into the blood till it had risen to near the top. The upper end was then sealed in a flame, care being taken not to burn the part containing blood.

The same centrifuge was always used at the same speed; the six tubes used in each determination were rotated for fifteen minutes, which was found sufficient to give approximately constant results.

The heights of the corpuscles and of the whole blood were then measured and the former expressed as a percentage of the latter. By using six tubes and taking the mean errors were minimised. The following figures illustrate this point, and give details of some other experiments which were done to test the validity of the method.

In one experiment after centrifuging for fifteen minutes a value of 47 per cent. was obtained. In a second experiment six tubes filled with the same blood were spun for seven and a half minutes, when a rather higher result was obtained—52·7 per cent. Obviously, sedimentation was not complete. The same tubes were then rotated for a second seven and a half minutes, when the result obtained was about the same as in the first experiment, *i. e.* 47·9 per cent. In a third experiment after rotating another six tubes with the same blood for fifteen minutes the result was 47·5 per cent. The same tubes were then spun for another ten minutes, when the value was only reduced to 46·9 per cent. In these three separate determinations on the same blood the values found were 47·0, 47·5, and 47·9.

In another experiment blood was taken from a subject on successive days with the following results: 34·5 per cent. and 36·3 per cent. A much greater difference than this was not found in any cases where separate determinations were made.

As regards the variations in individual tubes four consecutive experiments will give a good idea of this:

- i. 17·6, 16·2, 18·0, 21·5, 16·2, 17·6, 17·0, 18·8 = av. 18·0.
- ii. 70·9, 66·5, 68·2, 64·9, 67·4, 66·3, 66·1, 68·1 = av. 66·9.
- iii. 46·2, 43·6, 41·9, 35·5, 41·1, 38·9, 35·2, 36·7 = av. 40·0.
- iv. 28·2, 29·8, 31·1, 29·8, 26·7, 29·7, 32·9, = av. 29·7.

Obviously single determinations, which have usually been used by previous workers, could only give a rough indication, but the use of six tubes gives a more reliable figure. The reading obtained is accurate within 5 per cent.—an error greater than that of the Haldane-Gowers hæmoglobinometer and less than that of an ordinary red cell count.

Other experiments were done with capillary tubes which were obviously not of equal bore, in three tubes the corpuscles being at the broader end and in three at the narrower end. The results were as follows, in three separate experiments, six tubes being used each time: At the thick end, 35·1, 19·4, and 32·1; at the thin end, 39·1, 23·3, and 40·2. In the last case the different bore was very obvious, and in the two other cases the difference would have been recognised at once by inspection, and the tubes would not have been used in the ordinary routine.

One criticism which was urged against the use of this method was that the cells were subjected to too much pressure by rotating in the centrifuge. It was thought that no estimate of their true size could be obtained, and all that would be left would be the hæmoglobin and stroma, all fluid being forced out. To investigate this point the following experiment was performed. Some blood was centrifuged, and equal volumes of corpuscles were then mixed with equal volumes of 0·6 per cent. and 0·9 per cent. NaCl solutions. The former swelled up but hæmolysis did not take place. After standing for half an hour to reach equilibrium the mixtures were stirred up and sets of capillary tubes were filled with each mixture. These were centrifuged and measured in the usual way. In three experiments the following results were obtained with blood from different subjects; after mixing with 0·6 saline, 25·1 per cent., 15·0 per cent., and 37·2 per cent.; after mixing with 0·9 saline, 23·1 per cent., 12·6 per cent., and 31·6 per cent. In each case the blood with larger corpuscles gave a larger value for the relative volume of corpuscles, and in the third case, at any rate, the result was outside the limits of experimental error.

If the method is valid as a means of measuring the relative sizes of corpuscles which only differ as the result of osmotic change, it is certainly valid to measure variations in size, which depend on a more fundamental change in the nature of the corpuscles, as in pernicious and secondary anæmias. In any case the conditions observed in each experiment were strictly

the same, so that the compression during rotation would tend to produce a similar degree of change in each experiment. Even if the absolute size of the corpuscle is somewhat modified a comparison between the size in various conditions may still be made.

#### RESULTS.

Sixteen subjects with no anæmia were used as normal controls. The results are given in full in the appendix. The number of red cells, the hæmoglobin and the corpuscular volume are all expressed as percentages of the normal. In column 7 is given the "hæmoglobin index." It is obtained by dividing the hæmoglobin by the corpuscular volume and measures the concentration of hæmoglobin in the corpuscle.

*Secondary anæmia.*—In eleven cases of anæmia, mostly due to loss of blood, the red cells averaged 62 per cent., hæmoglobin 34 per cent. (12–56), and corpuscular volume 39·5 per cent. The colour index averaged 0·56 (0·38–0·83), and the volume index 0·66. The hæmoglobin index was 0·86—much nearer the normal than any of the other results. There is a reduction of about 30 per cent. in the average size of the red cells, but the concentration of hæmoglobin in the corpuscle is reduced only 10 per cent.

*Chlorosis.*—In eight cases of chlorosis the red cells averaged 70 per cent., the hæmoglobin 41 per cent. (19–68), and the corpuscular volume 51 per cent. The colour index averaged 0·56 (0·42–0·78), and the volume index 0·70. The hæmoglobin index was 0·80, relatively constant and only slightly below the corresponding figure for the secondary anæmias. The reduction in the size of the red cells is about the same.

*Pernicious anæmia.*—In seven cases of pernicious anæmia the red cells averaged 29 per cent.; hæmoglobin 34 per cent. (16–65), and corpuscular volume 41 per cent. Colour index averaged 1·24 and volume index 1·39. The hæmoglobin index averaged 1·00 and varied between 0·82 and 1·35. Thus the average size of the corpuscles was increased by 30 per cent. above normal, while the concentration of the hæmoglobin in each corpuscle was slightly (4 per cent.) above normal. In severe polycythæmia due to congenital heart disease, where the number of red cells is about doubled, there was no change in the colour index, in the size of the corpuscles, or in the concentration of hæmoglobin in the corpuscle. The same was found to be true in cases of acholuric jaundice. Taking these groups as a whole the most striking point is the relative constancy of the "hæmoglobin index." While the red count varies between 29 and 163 per cent. and the hæmoglobin between 34 and 140 per cent., the colour index varies between 0·56 and 1·20 and the volume index between 0·66 and 1·30, but the hæmoglobin index only varies between 0·80 and 0·96.

With changes of over 100 per cent. in the number of red cells and the hæmoglobin percentage, the colour index and size of the red cells vary over 66 per cent., but the concentration of hæmoglobin in each corpuscle only varies 16 per cent. It is almost, but not quite, true to say that the size of the corpuscle depends entirely on its hæmoglobin content.

If individual cases are considered instead of groups, the results are almost equally striking. The red cell count varies from 12 per cent. to 186 per cent., the hæmoglobin from 12 to 155 per cent., and the corpuscular volume from 15 to 176—very similar ranges of variation. The changes in the colour index



and the volume index are much less, but very considerable. The former lies between 0·38 and 1·5 and the latter between 0·46 and 1·80, or, if one case is excluded, 1·50.

But the hæmoglobin index which measures the concentration of hæmoglobin in the corpuscle only varies between 0·65 and 1·35. If four extreme cases are excluded—and two of these results are not considered equally reliable, as they were two of the six results obtained on capillary blood—the hæmoglobin index only varies between 0·75 and 1·15.

It is clear that in various pathological conditions the number of red cells is the most important factor in maintaining the hæmoglobin percentage; that the size of the red cells is the second most important factor; and that changes in the concentration of hæmoglobin in the corpuscle only occur to a slight extent and are relatively unimportant. The colour index is more nearly than had been supposed a measure of the average size of the red cells.

It is often stated that chlorosis is characterised by the particularly low colour index and by the pale corpuscles. The colour index in these groups is the same, and if each determination is plotted on a graph with colour index as ordinates and hæmoglobin percentage as abscissæ, there is in general a decrease of colour index with a decrease of hæmoglobin percentage, and the points representing chlorosis are not obviously lower than the points representing the secondary anæmias.

#### DISCUSSION.

*Corpuscular volume.*—In normal cases this varied between 32 and 45·6, but only in two cases was it below 36 and only in two cases was it above 42. It averaged 40 per cent. Capps (1903) found 48 per cent. and Larrabee (1911) 50 per cent., but the latter did not use a centrifuge, and the former did not add any reagent to prevent clotting.

Gram (1921) used a method more closely resembling this, and found that it varied closely with the hæmoglobin, the average result in normal cases being hæmoglobin 90 per cent. and corpuscular volume 42 per cent.

*Volume of the individual red corpuscle.*—In some ways this method is more accurate than the measurement of the diameter of the cells because it is the true volume which is measured, and such large numbers of cells are used and an average taken. A comparison between the two methods has been made by several observers.

Fischer (1915) found that  $6\cdot2\mu$  (diameter) corresponded to a corpuscular volume of 33·8;  $6\cdot4\mu$  to 35·0; and  $6\cdot7\mu$  to 39·0. Price-Jones (1920), using solutions of sodium bicarbonate, lactic acid and normal saline, found that a change in reaction produced changes in the size of the corpuscle. Averaging three of his separate experiments he found that  $6\cdot2\mu$  corresponded to 92 per cent.;  $6\cdot3\mu$  to 100 per cent.; and  $6\cdot5\mu$  to 113 per cent. Capps (1903) measured the mean diameter in a large number of pathological conditions, and found that it was  $8\cdot0\mu$  in cases of pernicious anæmia,  $7\cdot65\mu$  in normals, and varied between  $7\cdot1\mu$  and  $7\cdot5\mu$  in different groups of secondary anæmias and chlorosis. In pernicious anæmia Price-Jones found a mean diameter of  $8\cdot2\mu$  in dry films.

The diameter of the red cells in these cases was not measured, but suppos-

ing the increase took place equally in all diameters, an increase of 10 per cent. in volume is produced by an increase of just over 3 per cent. in diameter. Supposing there is not much change in the thickness of the corpuscle, an increase of 10 per cent. in the volume of the corpuscle would be produced by rather less than 5 per cent change in diameter, *e. g.* :

$$\text{If } v = kr^3 \text{ and } R = 1.033 r$$

$$V = kR^3 = k 1.10r^3 = 1.10 v,$$

$$\text{and if } v = Kr^3 \text{ and } R = 1.05 r$$

$$V = KR^3 = K 1.10 r^3 = 1.10 v,$$

where  $v$ ,  $r$ ,  $V$  and  $R$  are the volume and radius of the ordinary and of the larger corpuscle respectively. The true value must lie between these limits.

In this series the volume index was increased 30 per cent. in anæmias of high colour index and diminished 30 per cent. in anæmias of low colour index. This means that the mean diameter varied in these two groups by 9–15 per cent. from the normal. Taking  $7.7 \mu$  for the normal the mean diameter in these cases of high colour index would be about  $8.4 \mu$ , and in the cases of lower colour index about  $7.0 \mu$ .

All these methods agree in demonstrating the increased size of the average cell in pernicious anæmia in spite of the presence of microcytes. In chlorosis and secondary anæmias the average size of the red cells is reduced.

#### CONCLUSIONS.

(1) A method is described for estimating accurately the volume of red corpuscles in whole blood. In normals this was found to be 40 per cent.

(2) The corpuscular volume varies almost directly with the hæmoglobin percentage. There is little change in the concentration of hæmoglobin in the corpuscle in various pathological conditions.

(3) The term "volume index," which is the ratio of the corpuscular volume to the number of red cells, gives a measure of the relative size of the red cells. The size depends almost entirely on the hæmoglobin content. From (2) and (3) it follows that the colour index is a rough guide to the size of the average red cell.

(4) In pernicious anæmia the average cubic contents of each red cell is increased by about 30 per cent. In secondary anæmias and chlorosis it is diminished by about 30 per cent. In polycythæmia it is unchanged.

(5) The diameter of the corpuscles calculated from the volume agrees fairly closely with the direct measurements made by other observers.

(6) From many points of view the blood changes in chlorosis are exactly the same as in the secondary anæmias, and do not resemble in any way the changes in the other so-called primary anæmia.

Many of these conclusions follow from the very extensive investigations carried out by Capps, but his paper does not seem to have received the attention it deserves.

Gram was the first to point out clearly that the close parallelism between the colour index and the volume index indicated a constant concentration of hæmoglobin in the corpuscle: "Il en résulte que les écarts pathologiques de l'indice colorimétrique sont dus essentiellement à des variations du volume moyen des globules."



## APPENDIX.

1. —	2. Red blood- corpuscles, percentage of normal.	3. Hæmoglobin, percentage of normal.	4. Corpuscular volumes, Percentage of normal.	5. Colour index.	6. Volume index.	7. Hæmo- globin index.
1. Healthy student . . . .	114	86	104	0·75	0·91	0·83
2. " . . . .	118	99	107	0·84	0·90	0·93
3. " . . . .	118	86	105	0·73	0·89	0·82
4. " . . . .	106	84	82	0·75	0·73	1·02
5. " . . . .	108	96	92	0·83	0·79	1·04
6. " . . . .	98	96	92	0·98	0·94	1·04
7. " . . . .	110	87	97	0·79	0·88	0·90
8. Cerebral tumour . . . .	102	88	90	0·86	0·88	0·98
9. Carcinoma stomach . . . .	92	91	97	0·99	1·03	0·93
10. Cerebral hæmorrhage . . . .	102	108	101	1·06	1·00	1·06
11. Recovered chlorosis . . . .	100	85	97	0·81	0·94	0·81
12. Emphysema . . . .	96	95	90	0·99	0·94	1·06
13. Heart failure . . . .	84	86	90	1·02	1·07	0·95
14. Hodgkins' disease . . . .	102	90	101	0·88	0·98	0·90
15. Emphysema . . . .	90	82	80	0·90	0·89	1·01
16. Mitral stenosis . . . .	112	108	114	0·97	1·02	0·95
Average (1-16): 16 normals . . . .	103	92	100	0·89	0·97	0·96
17. Gallstones . . . .	86	70	77	0·81	0·90	0·91
18. Heart failure . . . .	66	70	70	1·06	1·06	1·00
19. Menorrhagia . . . .	84	42	50	0·50	0·60	0·84
20. Gastric ulcer . . . .	98	45	52	0·46	0·54	0·86
21. Sarcoma kidney . . . .	68	29	32	0·43	0·53	0·90
22. " . . . .	34	13	15	0·38	0·46	0·87
*23. Pyorrhœa . . . .	80	56	57	0·70	0·71	0·98
24. Gastric ulcer . . . .	46	33	34	0·72	0·74	0·97
*25. " . . . .	38	31	39	0·83	(1·04)	0·79
26. " . . . .	76	44	45	0·58	0·59	0·98
27. Chronic nephritis . . . .	64	39	47	0·61	0·71	0·82
28. Ulcerative colitis . . . .	70	32	42	0·45	0·60	0·75
*29. Carcinoma . . . .	30	12	18	0·40	0·62	(0·65)
Average (19-29): Secondary anæmia . . . .	62	34	39·5	0·56	0·66	0·86
*30. Chlorosis . . . .	98	50	62	0·50	0·62	0·80
31. " . . . .	90	51	59	0·56	0·65	0·86
*32. " . . . .	92	68	86	0·75	(0·92)	0·79
33. " . . . .	45	19	29	0·42	0·64	(0·65)
34. " . . . .	52	30	35	0·58	0·68	0·84
35. " . . . .	60	27	33	0·45	0·56	0·81
36. " . . . .	48	25	31	0·50	0·65	0·81
37. " . . . .	74	58	70	0·78	(0·94)	0·83
Average (30-37): Chlorosis . . . .	70	41	51	0·56	0·70	0·80
38. Infective endocarditis . . . .	34	34	36	1·00	1·06	0·94
39. Pernicious anæmia . . . .	32	35	35	1·12	1·10	1·00
40. " . . . .	18	27	27	1·50	1·52	1·00
41. " . . . .	23	25	27	1·08	1·20	0·91
42. " . . . .	12	16	18	1·34	1·50	0·94
43. " . . . .	20	27	31	1·35	1·55	1·15
44. " . . . .	48	65	87	1·36	1·80	1·35
45. Ditto (after transfusion) . . . .	50	48	59	0·96	1·18	0·82
Average (39-45): Pernicious anæmia . . . .	29	34	41	1·24	1·39	1·00

## APPENDIX—(continued).

1.	2. Red blood- corpuscles, percentage of normal.	3. Hæmoglobin percentage of normal.	4. Corpuscular volumes, percentage of normal.	5. Colour index.	6. Volume index.	7. Hæmo- globin index.
46. Congenital heart disease .	164	152	171	0.93	1.04	0.89
47. " " .	186	145	167	0.78	0.90	0.87
48. " " .	132	106	118	0.80	0.89	0.90
49. " " .	170	155	176	0.91	1.02	0.88
Average (46-49): Congenital heart disease	163	140	158	0.86	0.97	0.89
*50. Acholuric jaundice . .	36	38	30	1.06	0.85	(1.24)
51. " " . .	54	50	56	0.93	1.04	0.90
52. " " . .	70	62	74	0.90	0.90	0.84
Average (50-52): Acholuric jaundice .	53	50	53	0.97	0.93	0.97

*Present Series.*

Normals . . . . .	103	92	100	0.89	0.97	0.96
Secondary anæmia . . . .	62	34	39.5	0.56	0.66	0.86
Chlorosis . . . . .	70	41	51	0.56	0.70	0.80
Pernicious anæmia . . . .	29	34	41	1.24	1.39	1.00

*Capps (1903).†*

Normals . . . . .	96	86	95	0.90	0.99	0.91
Secondary anæmia . . . .	60	43	50	0.75	0.84	0.82
Slight anæmia . . . . .	87	70	82	0.85	0.95	0.89
Severe anæmia . . . . .	44	33	42	0.80	0.95	0.84
Very acute anæmia . . . .	50	28	36	0.60	0.72	0.83
Chlorosis . . . . .	81	49	66	0.66	0.82	0.80
" slight . . . . .	89	63	82	0.78	0.92	0.85
" severe . . . . .	74	36	53	0.54	0.72	0.75
Pernicious anæmia . . . .	29	32	40	1.27	1.37	0.92

*Gram (1921).*

Normals . . . . .	—	—	—	0.90	1.0	0.90
Anæmia of low colour index .	—	—	—	0.60	0.8	0.75
Pernicious anæmia . . . .	—	—	—	1.45	1.5	0.97

## NOTES TO APPENDIX.

\* The six results marked thus were obtained with capillary instead of with venous blood.

Six figures have been included in brackets where the result seemed to be well outside the range of variation for that group. In obtaining the average figure these results have all been included. They have not been included in the range of variation which is discussed in the text of the paper, especially as four of these six results were obtained on capillary blood.

† The colour index has not been calculated in exactly the same way, so that these results are not strictly comparable.

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## ON THE ANTITRYPTIC ACTION OF THE BLOOD.

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THE capacity of the blood-serum to inhibit the action of trypsin presents a problem requiring further investigation. The antitryptic power may be measured with a considerable degree of exactitude, and the interest of the function is increased by the fact that, while confined within certain limits in normal individuals, it is found to be enhanced beyond those limits in certain diseases. Since its first observation by Hahn in 1897, the inhibitory power has excited a good deal of attention from physiologists and pathologists, and several hypotheses have been put forward to explain its nature. It was at first assumed to be an evident immunity reaction, but later doubt was cast on this view, and various conceptions of a less complex character were adduced to explain the effect. The view of antitryptic action which appears to prevail at present, which may be called the physiological view, since it embodies the explanation of the phenomenon presented by Bayliss (1919) and by O. Cohnheim (1912), is that the reduction of tryptic action produced by serum is due to a diversion of the enzyme from the added substrate by the proteins of the serum, these proteins being assumed to be themselves acted upon by trypsin only slowly and with difficulty, but to be able to adsorb the enzyme and so to diminish its effective concentration. The implication of this conception is that there is no justification for regarding the antitryptic factor in the serum as an anti-enzyme; that the phenomenon is sufficiently elucidated by the interpretation of the manner of the inhibition which the conception affords, and that the only problem remaining for consideration lies, not in the inhibitory effect itself, but in the resistance of the serum-proteins to the action of the enzyme. This being so, the question becomes a purely physiological one and loses much of its interest to the pathologist. It is, however, my purpose here to suggest that the subject is not one to be lightly

dismissed as having been adequately explained by the hypothesis of simple adsorption, but that we have under consideration a problem of no small complexity and importance, and one from the further examination of which we may reasonably hope to increase our knowledge of the metabolic processes in health and disease. The main impediment to the acceptance of the simple explanation lies in the occurrence of the pathological variations. It is a fact that in certain conditions of disease the inhibitory power may depart appreciably, and often considerably, from the normal level, returning gradually to this level when the exciting factor has been removed, and any explanation of antitryptic action must take cognisance of this liability of the function to variation and must be compatible with it.

It will be apparent that there are several directions in which the further investigation of the problem might be undertaken. Having regard, however, to the hypothesis under consideration, it has seemed expedient in the first place to examine the relation existing between the serum-proteins and the antitryptic power. This examination has been undertaken by several investigators with regard to one point, namely, the association of the normal inhibitory action with the albumin and globulin fractions of the protein; but it does not appear to have been extended to the abnormal increment of the antitryptic factor, which would *à priori* necessarily be in this respect identical with the normal factor; nor does any inquiry appear to have been made as to whether there exists a quantitative relation between the serum-proteins and the antitryptic power. As these two points are also of importance in their bearing upon the nature of the inhibitory factor, the following observations are put forward as having been directed towards their elucidation.

#### METHOD.

A number of different procedures have been employed for the determination of the antitryptic power of the serum, all of which fall, however, into one of two categories, based on two different principles. In the methods of the first category, serum is added to an arbitrary excess of trypsin in the presence of a suitable substrate, and the amount of resultant digestion is determined in comparison with the amount produced by the same quantity of enzyme in the absence of the serum. Most of these methods require relatively large quantities of the reagents, including the serum, and although undoubtedly measuring the amount of inhibition under the conditions employed, are not necessarily to be regarded as affording an expression of the true quantitative relation between the enzyme and the inhibitory factor. The methods of the second category constitute practically a titration of the inhibitory factor against the trypsin. In these, graduated dilutions of the trypsin solution are mixed with uniform quantities of the serum under examination; a suitable substrate is then added, incubation maintained for a fixed period, and the result read by noting the presence or absence of an observable degree of digestion in the tubes. The requirement of the substrate is that it should afford readily observable evidence of digestion, and various substances have been used for this purpose; but the most generally useful I have found to be a 1 per cent. solution of caseinogen. This gives a heavy precipitate on acidification, but it



is readily converted by trypsin into a form in which the precipitate is no longer produced, and the solution after the addition of acid remains clear. If the serum under examination be diluted to 1 part in 10, and the graduated dilutions of trypsin employed be made to differ by one-tenth of their value, a very sharp end-point will be obtained, and the inhibitory power of different samples of serum may be readily compared and expressed in terms of the quantity of the given trypsin solution that has been neutralised by each. This method, which was originally devised by Fuld, and independently by Gross,\* has been adversely criticised, but I have found it constant in its results and applicable to small quantities of serum. The measurements obtained have, of course, been only comparative, but have been adequate for the purpose of the investigation.

#### THE RELATION OF THE NORMAL ANTITRYPTIC FACTOR TO THE PROTEINS OF THE SERUM.

The investigations of Cathcart (1904), Doblin (1916), Kurt Meyer (1911) and Teale and Bach (1920) have shown that the greater part of the antitryptic power lies in the albumen-fraction of the serum-proteins, Cathcart concluding that the whole of the inhibitory factor is in this fraction and that the globulin is not antitryptic. My own experience has indicated that although the partition of the inhibitory effect between the two fractions is not uniform throughout the various animal species, the albumen is always more inhibitory than the globulin, and that in the case of human serum the association of the effect with the albumen-fraction is very pronounced. The difference between the two fractions in this respect is shown in Table I.

#### THE QUESTION OF WHETHER THE ASSOCIATION OF THE INHIBITORY FACTOR WITH THE ALBUMEN-FRACTION APPLIES TO THE PATHOLOGICAL INCREMENT OF ANTITRYPSIN.

The protein-fractions were prepared by precipitation with ammonium sulphate. After half-saturation with this salt the globulin was filtered off, redissolved, and again precipitated. The two filtrates from the globulin were completely saturated with the salt, and the albumen separated by filtration. Each precipitate was then dissolved in such a volume of saline that the solution corresponded to a definite dilution of the original serum, and the strength of the solutions will be expressed in this way. Thus, a 1 in 10 solution of albumen will denote the albumen of 1 c.c. of serum dissolved in such a volume of saline as to give a final volume of 10 c.c. The solutions necessarily contained a certain amount of ammonium sulphate, which was not removed, as similar solutions of the salt alone were found to have no antitryptic action.

It will be seen in Table I that although practically the whole of the antitryptic power was in the albumen, the power of the albumen was less than that of the whole serum. This I have found to be constant. The loss is not, however, due to the absence of the globulin, since, in the case of human serum,

\* Described by von Bergmann and Meyer (1908). I have not adhered strictly to the original technique.

the inhibitory strength of the albumen is not increased by adding the globulin to it. The reconstructed serum thus formed has always inhibited the same amount of enzyme as the albumen alone. In the case, however, of those animals referred to above, in which the inhibitory power of the globulin is greater, the inhibitory power of the albumen is found to be increased by the addition of the globulin, but the mixture never attains the inhibitory power of the whole serum. The reduction is presumably due to an alteration in the albumen produced by the precipitation.

The question now under consideration is elucidated by Table I. It is seen there that the increment of the antitryptic factor in the abnormal sera B and D has the same relation to the protein-fractions as the normal factor in A and C. The "antitryptic indices" of the fractions are approximately the same as those of the whole sera, and the index B/A derived from the albumen is seen to be identical with that derived from the reconstructed sera made by mixing the two fractions. The slight discrepancy existing is probably due to experimental error, and is not altogether surprising, in view of the fact that a complicated quantitative procedure has been carried out on a small amount of material.

TABLE I.—*The Antitryptic Power of the Protein-Fractions of Sera having Different Inhibitory Capacities.*

	A.	B.	B/A.	C.	D.	D/C.
Serum 1 in 10 . . . . .	21	38	1·8	21	38	1·8
Albumen 1 in 10 . . . . .	17	29	1·7	19	32	1·7
Globulin 1 in 5 . . . . .	3	5	1·7	3	5	1·7
Albumen + globulin 1 in 10 .	17	29	1·7	—	—	—

A, B, C and D represent four samples of serum. The figures under A, B, C and D indicate the number of c.mm. of trypsin-solution just neutralised by 100 c.mm. of the solution of protein, 100 c.mm. of a 1 per cent. solution of caseinogen having been added as the substrate.

The figures do not suggest that the determination was always carried out to the nearest c.mm., but denote, in terms of whole numbers, the quantity of undiluted trypsin-solution in the logarithmic series of dilutions employed, these dilutions differing, as nearly as practicable, by one-tenth of their value.

The conclusion to be drawn is that, in human serum, the pathological increment is also contained almost entirely in the albumen-fraction of the serum protein, and that when two sera differ in their antitryptic power, the albumen-fractions differ in this respect to the same extent. The investigation of the quantitative relation between the serum-protein and the antitryptic power has, in consequence, involved a separation of the protein-fractions and a determination of the amount of each fraction in the various samples of serum examined.

#### THE QUESTION OF THE RELATION OF THE ANTITRYPTIC POWER TO THE QUANTITY OF ALBUMEN IN THE SERUM.

The relation of the antitryptic power to the quantity of albumen, and also to the quantity of globulin, is shown in Table II. In these observations the



determination of the protein was carried out by the Kjeldahl method, and herein an objection arose to the use of ammonium sulphate as the precipitant, since it was found very difficult to remove completely the salt from the filters. Hence, magnesium sulphate was used to separate the globulin, as, although it has been shown that the quantities of protein precipitated by saturation with magnesium sulphate and by half-saturation with ammonium sulphate are not necessarily identical, the agreement was thought to be sufficiently close for the purpose of the investigation. The globulin was, therefore, precipitated by magnesium sulphate and re-dissolved; and the separated fractions were coagulated by heat, filtered off, washed, and digested in sulphuric acid on the filter-papers. Only 1 c.c. of serum was used for this estimation, but the result was always controlled by the determination of the total coagulable protein in 0.5 c.c.

It will be seen from Table II that there is no proportional relation between the inhibitory power and the quantity, either of the total protein or of the albumen, but that the tendency is for sera with a high antitryptic index to have a low protein content and especially a low albumen content. The antitryptic power was, in these experiments, determined from the whole serum, but assuming the relative inhibitory capacities of the albumen-fractions to have been, as usual, the same as those of the whole sera, we see, on comparing the first sample with the last, that the albumen of the last had in unit mass nearly six times the inhibitory power possessed by the albumen of the first.

TABLE II.—*The Relation of the Antitryptic Power to the Quantity of Albumen and of Globulin in the Serum.*

Antitryptic index of serum.	Grm. in 100 c.c.		Per cent. of protein.		Total protein. (as control).
	Albumen.	Globulin.	Albumen.	Globulin.	
1.0	5.0	3.4	60	40	8.3
1.0	4.6	2.4	66	34	7.4
1.0	4.2	2.6	62	38	7.0
1.1	3.2	4.0	44	55	7.4
1.4	3.6	2.9	55	45	6.7
1.5	3.1	4.0	44	56	7.2
1.7	2.4	3.5	41	59	5.9
1.7	3.1	2.9	51	49	6.0
1.9	3.2	3.2	50	50	6.5
2.0	1.8	4.6	28	72	6.5

THE QUESTION OF THE RELATION BETWEEN THE ANTITRYPTIC POWER OF  
THE SEPARATED ALBUMEN AND THE CONCENTRATION OF  
THE ALBUMEN IN THE SERUM.

Here, in order to meet criticism on the ground that the relative inhibitory power of the different sera might not always be identical with that of their albumen-fractions, the albumen, as well as the globulin, was precipitated and redissolved, and the actual inhibitory power of the albumen was determined. It was, therefore, necessary to use ammonium sulphate as the precipitant, and

for this reason the amount of the fractions was obtained by centrifuging down, drying and weighing the coagulated proteins instead of by the Kjeldahl method. The results are shown in Table III:

TABLE III.—*Relation of the Antitryptic Power of the Serum-Albumen to the Quantity of Albumen in the Serum.*

Antitryptic index.	Albumen.	
	Grm. in 100 c.c. of serum.	
1·0	.	4·3
1·7	.	3·7
1·0	.	4·6
1·7	.	3·6

The solutions of albumen of which the inhibitory capacities were determined corresponded to definite dilutions of the sera from which they were derived, and had no relation to the quantity of albumen which they contained. This was ascertained only after the inhibitory power had been measured.

In the first pair of samples in Table III the albumen of the second serum has practically twice, and in the second pair more than twice, the inhibitory power of that of the first serum, when compared in terms of equal mass.

#### CONCLUSION.

In considering the bearing of these observations on the conception of the antitryptic action of the serum as a simple diversion of the enzyme from the added substrate by the proteins of the serum, we see at once that the conception in its simplest form has been invalidated by the already recognised fact that the inhibitory power is, in certain sera, practically limited to the albumen-fraction of the proteins. Obviously the diversion of the enzyme cannot be ascribed to the proteins as such. We have to recognise that there exists in this respect a difference between the more readily precipitable protein and the less readily precipitable protein, and that this difference is, in the case of human serum, a very significant one. The simplicity of the explanation is, therefore, already complicated by the fact that the inhibitory power of the albumen-fraction is found to be so much greater than the inhibitory power of the globulin-fraction. Evidently there exists some fundamental difference between the albumen and the globulin upon which this difference in their capacity for diverting trypsin is dependent, and the explanation, to be adequate, must elucidate this distinction. When we pass to a consideration of the pathological variation, the conception becomes still less satisfying. For not only can we not ascribe the inhibition to a diversion of the enzyme by the protein as such, but we can now not even ascribe it to a diversion by the albumen as such. We have seen that the antitryptic power does not vary with the concentration of albumen in the serum, but that, on the contrary, it may tend to vary inversely with that concentration—a serum with an abnormally large antitryptic power may have an abnormally small



albumen content. Hence, the explanation has to cover the fact that equal masses of albumen may divert widely different quantities of trypsin from equal quantities of substrate, and has to elucidate the distinction upon which this difference is dependent. In effect, the simple explanation will have to give way to a more complex one. That the inhibition is due to a process of adsorption is probable (Hedin, 1905, 1906<sup>1</sup> and <sup>2</sup>; Young, 1918), as it is probable that most of the reactions met with in the study of immunity are due to processes of adsorption; but this adsorption is evidently dependent upon something more than the fact that the serum contains protein. It is dependent upon some at present unrecognised character of this protein—a character which may under certain conditions vary in its intensity. This character may be no more than a certain degree of dispersion of the albumen, or it may be as obscure and incomprehensible as the character upon which antitoxic action depends; but there can be no doubt that its nature, and the nature of the stimulus to its variation in disease, still constitute an important problem in pathology.

#### SUMMARY.

(1) The albumen-fraction of the serum-proteins is more antitryptic than the globulin-fraction, and in human serum the inhibiting factor lies almost entirely in the former.

(2) This association of the antitryptic action with the albumen-fraction applies also to the abnormal increment of antitrypsin occurring in certain conditions of disease.

(3) There is no proportional relation between the antitryptic power of the serum and the concentration, either of the total protein or of the albumen; but on the contrary, an increased inhibitory capacity may be accompanied by a diminished protein content, and especially by a diminished albumen content.

(4) The antitryptic power of the serum cannot be conceived as due to a simple diversion of the enzyme by the protein as such, but must be dependent upon some at present unrecognised character of this protein.

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# THE SEROLOGICAL RELATIONSHIPS OF THE PARACHOLERA VIBRIOS TO *VIBRIO CHOLERÆ*, AND THE SEROLOGICAL RACES OF THE PARACHOLERA GROUP.

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IN 1918 two serological races of *Vibrio paracholerae*, associated with a limited choleraic outbreak in Egypt, were described by Mackie and Storer (1918), and for purposes of future reference were designated *V. paracholerae A* and *B*. These vibrios corresponded closely in general characters to known *V. cholerae* strains though differing from the classical cholera vibrio in being markedly hæmolytic on blood-agar plates; they were not agglutinated, even in low titres, by an antiserum to a known *V. cholerae* of epidemic origin, and agglutinating sera to both types failed to react with known *V. cholerae* strains. These paracholera strains were all highly virulent in animal experiment by intravenous and intraperitoneal injection. All the available evidence strongly supported their ætiological relationship to the choleraic disease with which they were associated. Similar organisms had been previously described and designated paracholera vibrios by Chalmers and Waterfield (1916), and by Castellani (1916).

In the original agglutination tests the serological differentiation of *V. paracholerae A* and *B* from one another and from *V. cholerae* was well marked by the technique employed:

The emulsions were prepared in plain 0·85 per cent. saline from 18–24 hours agar-slope cultures; a series of concentrations of the antiserum were tested, ranging from relatively low dilutions up to the end-titre for the homologous organism; the mixtures of diluted serum and emulsion (0·4 c.c. each) were placed in agglutination tubes 3 in.  $\times$   $\frac{1}{8}$  in., and the degree of agglutination was noted after two hours at 37° C. and also after 24 hours at room temperature. This technique had been extensively used by the writer, had always yielded uniform results, and had not exhibited apparent fallacies.

The question of serological races of *V. cholerae* has recently been studied by Douglas (1921). A number of strains from different sources were investigated and also certain non-cholera vibrios, including strains of *V. paracholerae A* and *B*. He concluded that *V. cholerae* represented one serological race and embraced also the hæmolytic varieties (corresponding to the El Tor vibrio)—conclusions also arrived at by the writer in the course of certain studies of *V. cholerae* and other intestinal vibrios in Egypt.

Douglas, using formolised broth cultures as emulsions for the agglutination tests (according to Dreyer's method) and incubating at 50°–55° C. for 2 hours, found that after continued subculture on artificial medium *V. paracholerae A* acquired the property of being agglutinated by anticholera sera, though without a corresponding result in absorption tests. Douglas also found that in the case of emulsions prepared in plain salt solution, and incubating at 50°–55° C.,



a *V. cholerae* serum agglutinated *V. paracholerae* A and B, and the results with known strains of *V. cholerae* were somewhat irregular.

Such results might be interpreted as indicating a less distinct serological difference of these paracholera types from *V. cholerae* than that originally claimed for these species, and are at variance with the writer's original observations.

To quote the results of the original tests carried out by the technique referred to; none of the paracholera strains were agglutinated by a 1:50 dilution of an anticholera serum (Lister Institute—1916) whose end-titre was 1:2000; the tests were repeated after the strains had been subcultured several times, with similar results, and the strains after recovery from experimental animals still failed to react with the anticholera serum. Absorption tests, Pfeiffer's reaction and complement-deviation tests yielded corresponding results. An antiserum also to a *V. paracholerae* A strain agglutinated homologous strains to a titre of 1:1600, but had no effect on a strain of *V. cholerae* or different strains of *V. paracholerae* B in dilutions of 1:100. An antiserum to a strain of *V. paracholerae* B agglutinated the homologous strains up to dilutions of 1:3200, but had no action on *V. cholerae* or *V. paracholerae* A strains in titres of even 1:100.

The marked differences between plain and formalised emulsions as indicated by Douglas seems a factor of the greatest importance in agglutination technique, apart from their significance in regard to the particular races under consideration. The opportunity, therefore, was taken of re-testing certain paracholera strains (still kept in culture by the writer) with an antiserum to a *V. cholerae* (Egyptian origin), especially as these strains had been continuously cultivated on artificial medium for a period of four to five years.

The agglutinating serum to *V. cholerae* was prepared in the usual way and tested with the homologous strain and a *V. paracholerae* A strain "G."

Careful comparisons were made between the results obtained when the tests were carried out by different methods. Different agglutination emulsions were compared: (1) plain saline emulsions from 18- to 24-hour agar slope cultures; (2) saline emulsions formalised—0.1 per cent. formalin; (3) formalised broth emulsions prepared by Dreyer's method. Further comparisons were also made between the results obtained after incubation at 37°C. and 55°C. for 2 hours, and readings were taken after the test mixtures had been standing at room temperature overnight following removal from the incubator.

The results are shown in Table I. Repetition of these experiments yielded similar and uniform results.

Thus, even when agglutination tests were carried out by a different technique, the effects after 2 hours were identical, *i.e.* the *V. paracholerae* A strain was not agglutinated by the *V. cholerae* serum even in low dilutions; "coagglutination" effects were evident after 24 hours with the plain saline emulsion incubated primarily for 2 hours at 55°C., the formol-saline emulsion and the formol-broth emulsion, though absent in the case of the saline emulsion incubated first for 2 hours at 37°C. These coagglutination effects were most marked in the case of the formol-broth emulsion incubated for 2 hours at 55°C., but in degree and end-titre always fell short of the specific agglutination of the homologous type.

TABLE I.—*V. cholerae Antiserum: Serum Dilutions.*

	1:200.	1:400.	1:800.	1:1600.	1:3200.	1:6400.	1:12,800.	Control.
<i>Saline emulsions:</i>								
<i>V. cholerae:</i>								
2 hours 37° C.	+++	+++	+++	+++	+++	++	—	—
24 hours room temperature.	"	"	"	"	"	++	—	—
<i>V. paracholerae A:</i>								
2 hours 37° C.	—	—	—	—	—	—	—	—
24 hours room temperature.	—	—	—	—	—	—	—	—
<i>V. cholerae:</i>								
2 hours 55° C.	+++	+++	+++	+++	+++	+++	++	—
24 hours room temperature.	"	"	"	"	"	++	—	—
<i>V. paracholerae A:</i>								
2 hours 55° C.	—	—	—	—	—	—	—	—
24 hours room temperature.	+++	++	—	—	—	—	—	—
<i>Formol-saline emulsions:</i>								
<i>V. cholerae:</i>								
2 hours 37° C.	+++	+++	+++	+++	+++	—	—	—
24 hours room temperature.	"	"	"	"	++	++	—	—
<i>V. paracholerae A:</i>								
2 hours 37° C.	—	—	—	—	—	—	—	—
24 hours room temperature.	+++	++	—	—	—	—	—	—
<i>V. cholerae:</i>								
2 hours 55° C.	+++	+++	+++	+++	+++	—	—	—
24 hours room temperature.	"	"	"	"	++	++	—	—
<i>V. paracholerae A:</i>								
2 hours 55° C.	—	—	—	—	—	—	—	—
24 hours room temperature.	+++	++	—	—	—	—	—	—
<i>Formol-broth emulsions:</i>								
<i>V. cholerae:</i>								
2 hours 37° C.	+++	+++	+++	+++	+++	—	—	—
24 hours room temperature.	"	"	"	"	++	++	—	—
<i>V. paracholerae A:</i>								
2 hours 37° C.	—	—	—	—	—	—	—	—
24 hours room temperature.	+++	+++	+	+	—	—	—	—
<i>V. cholerae:</i>								
2 hours 55° C.	+++	+++	+++	+++	+++	—	—	—
24 hours room temperature.	"	"	"	"	"	+	—	—
<i>V. paracholerae A:</i>								
2 hours 55° C.	—	—	—	—	—	—	—	—
24 hours room temperature.	+++	+++	+++	+++	+++	++	—	—
<i>V. cholerae:</i>								
2 hours 55° C.	—	—	—	—	—	—	—	—
24 hours room temperature.	+++	+++	+++	+++	+++	++	—	—
<i>V. paracholerae A:</i>								
2 hours 55° C.	—	—	—	—	—	—	—	—
24 hours room temperature.	+++	+++	+++	+++	+++	—	—	—

Degree of agglutination indicated by the number of + marks; — signifies absence of agglutination.

"24 hours room temperature" refers to readings made after the tubes had remained at room temperature for 24 hours following incubation at 37° C. or 55° C.



It was thus apparent that the coagglutination effect of the *V. cholerae* serum with the *V. paracholerae A* strain was (1) a late effect only developing slowly and therefore not comparable with the specific reaction, (2) of lesser degree and lower end-titre than the reaction with *V. cholerae*, (3) more markedly elicited by certain methods, particularly when formol-broth emulsions were used and incubated first at 55° C.

The results in no way disprove the original claim that *V. paracholerae A* was sharply distinguished in direct agglutination test from *V. cholerae* (apart altogether from the results of absorption tests). These observations again show that the sharpest distinctions are obtained by using plain saline emulsions and incubating at 37° C.

A strain of *V. paracholerae B* ("L") tested with the same dilutions of the anticholera serum even in the case of formol-broth emulsions and after primary incubation at 55° C. showed no coagglutination in the 24 hours' readings (Table II). This was also noted in Douglas's experiments.

Three other paracholera vibrio strains originally isolated from choleraic cases in the Egyptian Expeditionary Force, each representing different serological types (not *A* and *B*), were also tested in comparative experiment, using plain saline emulsions and formol-broth emulsions. The results are shown in Table II and correspond with those elicited with *V. paracholerae A*.

It is also of interest to refer to and summarise here certain observations carried out by the writer in Egypt in 1916-1918 on the question of serological classification in the group of intestinal vibrios biologically allied to *V. paracholerae A* and *B*.\*

Altogether 57 strains of this group were studied corresponding in general characters to *V. paracholerae A* and *B* as originally described; these were isolated from typical choleraic cases, from cases of non-choleraic diarrhoea, and from healthy carriers (including contacts of choleraic cases and cases convalescent from "cholera" and acute diarrhoea).

The 57 strains were all classified serologically, and among them 20 different serological types (including *A* and *B*) were identified. Eight of the serological types were represented in the series by one strain only, though the other types included multiple representatives.

The serological differentiation of these races from *V. cholerae* and from one another was well marked, as in the case of *V. paracholerae A* and *B*. The antiserum to one type agglutinated organisms of the same type, the end-titre corresponding to that for the strain used in immunisation, but had practically no effect on strains of other types even in low titres.

It was assumed from the evidence available that vibrios of this group were responsible for a condition varying from simple diarrhoea to an acute illness clinically indistinguishable from Asiatic cholera.

The paracholera cases were for the most part sporadic or occurred in the form of limited "outbreaks."

It was impossible to establish any distinction in the types of cases associated with the different races. Only two fatal cases were noted in the series—the associated vibrios representing different serological types. Certain of the

\* These investigations have not been published owing to the loss at sea in 1918 of the detailed records bearing on this work.

TABLE II.—*V. cholerae* Antiserum: Serum Dilutions.

<i>Saline emulsions:</i>		1:200.	1:400.	1:800.	1:1600.	1:3200.	1:6400.	1:12,800.	Control.
<i>V. cholerae:</i>	2 hours 37° C.	+++	+++	+++	+++	+++	++	—	—
	24 hours room temperature	"	"	"	"	+++	++	—	—
<i>V. paracholerae</i> B:	2 hours 37° C.	—	—	—	—	—	—	—	—
	24 hours room temperature	—	—	—	—	—	—	—	—
<i>Vibrio "K":</i>	2 hours 37° C.	—	—	—	—	—	—	—	—
	24 hours room temperature	—	—	—	—	—	—	—	—
<i>Vibrio "Forrest":</i>	2 hours 37° C.	—	—	—	—	—	—	—	—
	24 hours room temperature	—	—	—	—	—	—	—	—
<i>Vibrio "Belah":</i>	2 hours 37° C.	—	—	—	—	—	—	—	—
	24 hours room temperature	—	—	—	—	—	—	—	—
<i>Formol-broth emulsions:</i>									
<i>V. cholerae:</i>	2 hours 55° C.	+++	+++	+++	+++	+++	+	—	—
	24 hours room temperature	"	"	"	"	+++	+	—	—
<i>V. paracholerae</i> B:	2 hours 55° C.	—	—	—	—	—	—	—	—
	24 hours room temperature	—	—	—	—	—	—	—	—
<i>Vibrio "K":</i>	2 hours 55° C.	—	—	—	—	—	—	—	—
	24 hours room temperature	+++	+++	+++	+++	++	—	—	—
<i>Vibrio "Forrest":</i>	2 hours 55° C.	—	—	—	—	—	—	—	—
	24 hours room temperature	+++	+++	+++	+++	++	—	—	—
<i>Vibrio "Belah":</i>	2 hours 55° C.	—	—	—	—	—	—	—	—
	24 hours room temperature	+++	+++	+++	+++	++	—	—	—



types including *A* and *B* were associated with severe choleraic cases, but strains of the same types were also isolated from non-choleraic diarrhoea and from healthy carriers. Some types were only associated with simple diarrhoea, and others were represented only by strains isolated from healthy carriers. These vibrio carriers were cases convalescent from "cholera" or acute diarrhoea, or men who had been in "contact" with choleraic cases though themselves presenting no history of intestinal illness.

The large number of different serological types encountered in proportion to the total number of strains examined raised the question of the actual significance of such wide serological differentiation.

In the *B. coli* group it has been noted (Mackie, 1913) that agglutinating anti-sera show a highly restricted specificity, *i. e.* for the individual strain. A closely analogous condition as regards serological reactions has also been observed in the group of hæmophilic bacteria (Maitland and Cameron, 1921), serologically identical strains being infrequent. Among the series of paracholera vibrios investigated the majority of the types recognised were represented by multiple strains. Types *A* and *B* embraced the largest number of strains in the series.

Strains of *V. paracholerae A* and *B* were isolated in different places and at intervals of six months. Dr. Ledingham, while Consulting Bacteriologist to the Mesopotamia Expeditionary Force, informed the writer that he had examined a vibrio strain in Mesopotamia in 1917 corresponding to the *B* type (identified by agglutinating antiserum supplied to him from Egypt). Moreover the "*V. Gindha*" of Chalmers and Waterfield isolated at Port Sudan in 1915 was found to be serologically identical with two strains isolated in 1917 in the Egyptian Expeditionary Force. It is also of interest to note that Strain 10 El Tor, in the serological experiments carried out recently by Douglas, behaved like *V. paracholerae A*.

It can hardly be said that the serological reactions in this group are analogous to that in the *B. coli* group or the group of hæmophilic bacteria; on the other hand, these organisms cannot be classified into a relatively small number of serological types as in the case of certain bacterial groups. It is apparent that no definite conclusions regarding the significance of the serological differentiation noted in this group could be arrived at without a more extensive study of these vibrios and the examination of a large number of strains, both from the standpoint of their ætiological relationships and their serological reactions. The observations summarised here, though somewhat limited, represent a further contribution to the study of serological classification among the bacteria, apart from their particular interest in connection with the group studied.

#### CONCLUSIONS.

(1) The paracholera vibrios comprise a group which is not serologically homogeneous, but, in addition to *V. paracholerae A* and *B* already described, represents a considerable number of serological races precisely differentiated by agglutination reactions.

(2) By direct agglutination tests, using plain saline emulsions and incubating

at 37°C. for 2 hours, the paracholera vibrios are distinctly differentiated from *V. cholerae*.

(3) *V. cholerae* antiserum exhibits apparent co-agglutination under certain conditions towards *V. paracholerae* A and certain similar types; this effect develops more slowly than the agglutination of the homologous organism and is of lesser degree and of lower end-titre; it is most markedly elicited when formol-broth emulsions are used and the tubes are incubated first at 55°C.

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## THE ANTI-BACTERICIDAL PROPERTIES OF COLLOIDAL SILICA.

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DURING the course of an investigation into the anti-bactericidal action of the bile salts in connection with the culture of *B. typhosus*, the author (1911) was led to the conclusion that bile owes its power of assisting the growth of typhoid bacilli in blood cultures to its power of inhibiting the action of "complement." It appeared likely that this anti-complementary action of bile and the bile salts was attributable to their colloidal characters when in solution or perhaps when their solutions were mixed with body fluids, but this point was not definitely settled, and it was hoped that work on similar lines with known colloidal solutions might throw further light on the question. Some experiments with silica were actually contemplated in 1914, but the outbreak of war rendered them impossible. It was therefore with great interest that the author heard of the work of W. E. Gye and E. H. Kettle, of which mention was made by Dr. H. H. Dale (1921), since it appeared possible that the adjuvant action of colloidal silica in regard to the establishment of the tubercle bacillus in the tissues of experimental animals might be explicable in terms of the inhibition of bactericidal activity at the site of inoculation. On putting these suggestions to Dr. Gye early in the current year, he was so kind as to furnish the author with a supply of colloidal silica for the purpose of testing the point by experiment.

Since the inception of these investigations, my attention has been called



to a paper by Mutermilch and Bankowski (1913), who showed that both colloidal silica and also colloidal alumina have the property of adsorbing "complement," conclusions which my experiments confirm.

Further communications of the highest interest by Gye and Purdy (1922) have since appeared upon the poisonous effects of colloidal silica.

The importance thus given to this substance in relation to infection and resistance seems to justify the publication of a note on the anti-bactericidal properties of colloidal silica.

My thanks are due to Dr. W. E. Gye and Dr. H. H. Dale, F.R.S., for their kindness in providing me with a supply of colloidal silica, and to Miss C. M. Acland for her help in carrying out the experiments.

#### THE EFFECT OF COLLOIDAL SILICA UPON THE ANTI-BACTERIAL ACTION OF BLOOD.

*Experiment 1.*—5 : 4 : '22. A 24-hour broth culture of *B. typhosus* was diluted with sterile normal saline as follows :

(1) 1/100 . (2) 1/1000 . (3) 1/10,000 . (4) 1/100,000 . (5) 1/1,000,000.

Two series of Dreyer's tubes, each five in number, were prepared, containing the following reagents :

##### Series A :

1 vol. distilled water.

1 vol. typhoid emulsion (from dilutions 1 to 5).

2 vols. fresh blood (S.L.C.)

##### Series B :

1 vol. silica colloid.

1 vol. typhoid emulsion (from dilutions 1 to 5).

2 vols. fresh blood (S.L.C.).

It will be seen that, in each series, each tube from 1 to 5 contained a similar amount of fresh active blood, together with a measured volume of typhoid emulsion in which the number of bacilli diminished in tenths; the only difference being that in Series A, there was no silica colloid, but only a volume of distilled water, while, in Series B, each tube contained a volume of silica colloid.

Both series were now placed in the incubator at 37° C. for 1 hour 40 minutes. They were then withdrawn and, from each tube, a measured volume was abstracted after shaking and added to the surface of an agar slope, the agar cultures being numbered, in each series, from 1 to 5. These agar cultures were now incubated over night and the growths examined.

The results were as follows :

	Tubes (1) 1/100	(2) 1/1000	(3) 1/10,000	(4) 1/100,000	(5) 1/1 million.
Series A (with- out silica) .	2 colonies	1 colony	sterile	sterile	sterile
Series B (with silica) .	Colonies	Colonies	129	19	4
	innumerable	innumerable	colonies	colonies	colonies

This experiment makes it clear that, while in Series A there was normal

bactericidal activity, as shown by the almost complete destruction of typhoid bacilli within the space of 1 hour 40 minutes, in Series B, the presence of the silica colloid was sufficient to preserve the bacteria from the action of the blood.

*Experiment 2.*—12 : 4 : '22. This experiment was a repetition of Experiment 1 except that a stronger culture was used, trypsin broth being substituted for the bouillon previously employed. Also, to make sure that there should be no fallacy introduced by retention of bacilli in clot, the whole contents of each tube, including the small clotted residue, was transferred to the surface of the agar slopes and carefully spread with a small platinum spatula. The results were as follows :

	Tubes (1) 1/100	(2) 1/1000	(3) 1/10,000	(4) 1/100,000	(5) 1/1 million.
Series A (with- out silica)	Many discrete colonies	About 250 colonies	About 80 colonies	10 colonies	2 colonies.
Series B (with silica)	Continuous film of growth	Continu- ous film	Continu- ous film	Innumerable discrete colonies	Many discrete colonies.

These two experiments, the second completely confirmatory of the first, make it evident that colloidal silica is very active in arresting the action of the bactericidal substances of the blood ; and perhaps indicate to some extent what may take place when, as discovered by Gye, the addition of silica to a bacterial suspension enables the germs to establish themselves without check on inoculation into an experimental animal.

They suggest, too, that colloidal silica may offer a very promising medium for blood culture for organisms of the typho-colon group ; and perhaps for other pathogenic organisms.

#### THE EFFECT OF COLLOIDAL SILICA UPON "COMPLEMENT."

It was now time to ascertain, if possible, whether this "anti-bactericidal" action of silica colloid was due, as in the case of the bile salts, to interference with the action of "complement."

*Experiment 3.*—A mixture of three parts silica colloid with one part of a 3·2 per cent. sodium chloride solution was prepared so as to obtain a salt content of 0·8 per cent. in the silica mixture. It may be noted in passing that this mixture, while remaining fluid during the period of the experiment, was found some days later to have assumed the consistency of a gel.

Two series of small test tubes were placed in order, each consisting of five tubes.

Reagents were added as follows :

Series A.—To each tube—

0·1 c.c. of a 5 per cent. suspension of sensitised red blood-cells.

0·1 c.c. of sterile normal salt solution.

Series B.—To each tube—

0·1 c.c. of 5 per cent. suspension of sensitised red blood-cells.

0·1 c.c. of silica colloid with 0·8 per cent. salt.



To the tubes in each series, in order from 1 to 5, was now added complement in the following amounts:

(1) 0.2 c.c. . (2) 0.3 c.c. . (3) 0.4 c.c. . (4) 0.5 c.c. . (5) 0.7 c.c.

The total volume in each tube was now raised to 1 c.c. by the addition of sterile normal saline solution.

The results were as follows:

	Tubes (1)	(2)	(3)	(4)	(5)
Series A (without silica)—					
Hæmolysis .	Trace .	Some .	Marked .	Nearly complete	Complete
Series B (with silica)—					
Hæmolysis .	None .	None .	None .	None .	None

From this experiment, it was clear that the action of the "complement" had been completely inhibited by the addition of silica colloid to the mixture.

This experiment was repeated on several occasions with the same result, both with the sample of silica colloid received from Dr. Gye and a fresh sample kindly provided by Dr. Dale.

It was further ascertained that the solution was active after heating to 100° C. in steam for 30 minutes and that, while one volume of a ten-fold dilution of the colloidal silica could completely inhibit the action of two minimal hæmolytic doses of "complement," this inhibitive action was not demonstrable when the silica was diluted one hundred fold or upward.

The method of preparation of silica colloid is given by Gye and Purdy in their paper already quoted. In my experiments, the solution used was the water-clear solution, prepared by adding a dilute solution of sodium silicate to excess of strong HCl and then freeing the mixture of the HCl and NaCl by dialysis as described by these authors.

#### CONCLUSIONS.

1. Colloidal silica has the property of interfering with the bactericidal power of blood in marked degree.

2. It inhibits the action of "complement" in a hæmolytic mixture. This anti-complementary property is probably the character to which it owes its anti-bactericidal power.

3. These properties, together with its want of any demonstrable germicidal power, point to colloidal silica as likely to be of great use in blood cultures in cases of bacteriæmia.

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## SILICOSIS AND MINERS' PHTHISIS.

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It has long been recognised that certain occupations predispose to pulmonary tuberculosis, and that in these "dangerous" occupations the death-rate from this disease is far higher than in the general population. This is particularly so among those who work habitually in atmospheres which are laden with silica dust. The silicotic lung itself is a well-recognised pathological entity, presenting, in addition to the general increase in bulk of the connective-tissue trabeculae common to all forms of pneumoconiosis, the development of nodules or groups of nodules of dense fibrous tissue which often surround visible particles of silica. This condition, in itself sufficiently serious, is, however, of immeasurably greater importance from the fact that the silicotic lung is far more vulnerable to invasion by bacteria, and especially by the tubercle bacillus, than the normal organ. The general facts which have been accumulated, and upon which these conclusions are based, have been summarised by Collis in the Milroy Lectures for 1915. The association of tuberculosis with silicosis in Cornwall has been established by Haldane (1904); in South Africa by Watkins Pitchford (1916) and his collaborators. Experimentally, Gardner (1920) has been able to show that a dust containing a large percentage of silica influences profoundly the course of a tuberculous infection of the lungs of guinea-pigs. Gardner used a strain of tubercle bacillus of such low virulence that "guinea-pigs infected by inhalation of this strain show lung tuberculosis, which, however, clears up with restoration of function." When guinea-pigs were dusted with a granite dust and infected with tubercle, the lungs, instead of showing "a compact, sharply circumscribed tubercle," show "a central solid mass with heavy radiating peripheral processes." There is an early and progressive fibrosis in the dusted animals which tends to retard the healing of the tuberculous lesions.

Thus, clinically, there is overwhelming evidence that silica induces a fibrosis of the lungs, which are then sensitive to tuberculosis; and experimentally there is sound evidence supporting the clinical conclusions. The explanation of these facts is yet to be sought. The greater part of the experimental work which has been done on the subject has been concerned with the mode of entry of the dust into the lungs; the literature on this is very large, and the reader is referred to Collis' lectures and to Willis (1921) for the bibliography. It may now be said that there is general agreement that the very fine particles of dust which reach the lung alveoli are taken up by phagocytes, which, according to most authors, are derived from the endothelium of the lung capillaries, and, according to others, from alveolar epithelium, and that the dust-laden phagocytes migrate to the nearest lymphoid tissue and gain access to the closed lymphatic vascular system which drains

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the lung. Most dusts are removed from the lung in this way through the lymphatic channels, but silica, for the most part, remains *in situ*, stimulating the formation of new connective tissue.

The two problems arising out of the recorded knowledge which have occupied our attention during the last two years are:

- (1) Why is silica, conspicuous and possibly alone among common dusts, capable of inducing extensive fibrosis?
- (2) How does the silicotic fibrosis aid the establishment of tuberculosis?

(1) There have been many attempts made to answer the first question. For the most part, however, the answers have not been based upon experimental study. The view most often expressed is that silica owes its injurious quality to its physical properties (Watkins Pitchford, Oliver, Moore and others). Moore (1918) tabulates the properties as follows: "(1) Its heaviness; (2) its sharp vitreous fracture; (3) its great hardness and insolubility." Hardness, heaviness and sharpness of points or edges are properties which lose their significance when applied to particles of dust enclosed in the cytoplasm of wandering cells; direct mechanical injury to the lung (in the circumstances under which dust gains access) is almost inconceivable. The property of insolubility deserves more attention. Silica ( $\text{SiO}_2$ ), though insoluble in all acids except hydrofluoric, is readily dissolved in alkalis. By boiling finely divided silica in a solution of  $\text{Na}_2\text{CO}_3$  sodium silicate is formed. The statement, therefore, that silica dust is insoluble is only partially true, and the conditions under which a solution may be obtained are relevant to the problem of silicosis. Carbon particles—certainly in the test-tube—are more insoluble than silica particles, and it is universally agreed that carbon dust, unless in large amounts, is of no pathological importance. The suggestion, therefore, that an inhaled dust may, *in virtue of its insolubility*, exert a harmful action on the tissue is contrary to observed facts. Moreover, it is contrary to modern conceptions of the energetics of the body. In silicosis the injury to the lungs is a continuous process, and the amount of fibrous tissue formed is much greater than can be explained upon any mechanical process involving disturbance of microscopic anatomical relationships. Further, the fibrosis extends far beyond the near neighbourhood of the silica particles. In order that a continuous reaction between cells and a dust should ensue, molecular or submolecular contact must be established. Just as, in a physiological sense, no material is of use for energy purposes unless it is soluble, so, pathologically, with certain obvious mechanical exceptions, it is only soluble substances which can exert a substantial or a continuous effect on the tissues.

Haldane (1918) offers another answer to the question, "Why is pure quartz so difficult to remove from the lungs?" He supposes that coal-dust, shale, etc., owe their properties to a high adsorptive power, of which silica possesses little or none. Thus dust particles are attractive to dust-cells in proportion to the soluble substances adsorbed. The explanation is, therefore, that silica, adsorbing little or none of the substances which stimulate phagocytosis, is not removed from the lungs. But as we know that the way by which silica is carried into the lymphatics of the lungs is by the dust-cells this answer appears to us to be unsatisfactory.

Mellor (1918), in a written contribution to the discussion on Haldane's paper, draws attention to the surface of the small particles of silica. He states that "the surface of the fine grains of silica was sometimes found to be corroded, so that the surface appeared to be covered with a film crust of colloidal silica." It has been shown that colloidal silica is a toxic substance (Gye and Purdy, 1922), and Mellor's observation may, therefore, provide an answer to the problem which is the reverse of Haldane's, namely, that the film of colloidal silica on the particles, whilst not preventing phagocytosis, does diminish the mobility of the phagocytes, which are slowly poisoned. Mavrogordato (1922) shows that the dust-cells are immobilised in the lymphatic channels, and suggests that they become fixed and preserved by the action of the silica which they carry. Collis includes in the list of properties of silica dust, which may be significant in the solution of the problem of silicosis, the chemical property of "acidity, which, owing to the presence of the element silicon, may render the particles capable of entering into and modifying the colloidal structure of protoplasm." The meaning of this is not clear. The trend of opinion, therefore, is in favour of the view that silica causes fibrosis by acting chemically upon the tissues, and not in virtue of its physical properties.

(2) The second problem is even more difficult, and much less work has been done on it. The disorganisation of the lymphatic system in silicosis has been recognised by most authors as a potent factor in the increased susceptibility to tuberculosis. Gardner accepts this view, but adds a further explanation of the prevalence of tuberculosis among the victims of silicosis. He regards silica dust and the tubercle bacillus as irritants, and the two "acting together are capable, in a short time, of setting in motion a series of reactions on the part of the lung which neither alone is capable of initiating. By concentration of both in small foci each reinforces the action of the other, resulting in a chronic lesion which is slow to heal." Our own work on this question is the outcome of investigations into the factors influencing infection and immunity which have occupied our attention for some time. Among other substances we have used silica as a means of breaking down the normal resistance of tissues to invasion by micro-organisms, and while working with *B. tuberculosis* we obtained results which seem to have a direct bearing upon the pathology of miners' phthisis, and suggested further inquiries along these lines. The principal element in the problem of miners' phthisis is that men who are resistant to tuberculosis become susceptible with the development of silicotic fibrosis. It is important to understand the mechanism of this increased susceptibility, not merely as a part of the problem of industrial tuberculosis, but also because general tuberculosis presents a similar though more subtle problem.

Our work has been based on the known facts—(1) that colloidal silica is a cell poison; (2) that colloidal silica is the most easily formed soluble form of silica, the circulation of silica in nature depending upon this; and (3) that living matter (soil bacteria) is able to break up mineral silicates with formation of soluble silica (Bassalik, 1913). We judged it probable that the fibrosis brought about by finely-divided silica was due to the *slow* formation in the tissues of silica sol, either directly or through the intermediate formation of sodium silicate which is decomposed by carbonic acid, the sol formed acting as



a cell poison. The direct proof of this has been found impossible, and we are, therefore, compelled to fall back upon indirect evidence. The considerations which we think rule out the "physical" explanation of silicosis have already been given; the indirect evidence which supports our hypothesis is given below.

*Experimental.*—In approaching experimentally a subject of this complexity it is obviously necessary to proceed from the most simple experiments to those in which several factors are concerned, and we have attempted to clear the ground by studying the effects of pure silica ( $\text{SiO}_2$ ), of a powder containing 40 per cent. water and of silica sol in the subcutaneous tissue. This has been followed by experiments in which tubercle bacilli have been injected in association with silica or with an inert substance (carbon). Mice were chosen as experimental animals in the earlier work, partly because of their convenience as to size, etc., but chiefly because they possess a high degree of resistance to tuberculosis, though the resistance appears to be rather different from that exhibited by man.

Our technical methods have differed from those adopted by most workers in this field. We have used subcutaneous inoculations rather than dust inhalations, because of the greater ease with which local reactions could be followed.

#### THE RESULTS OF INJECTING SILICA INTO THE SUBCUTANEOUS TISSUE.

In these experiments the soluble colloidal form of silica, orthosilicic acid  $\text{Si}(\text{OH})_4$  and the insoluble silica ( $\text{SiO}_2$ ) were dissolved or suspended in normal neutral salt solution, and injected in doses of 2 mgrm. into the subcutaneous tissue of the flank. For the most part mice were used in these experiments, but from time to time results were compared with similar lesions produced in guinea-pigs and rabbits. The animals were killed at stated times, the local lesions carefully dissected out and fixed in Zenker's fluid or formol salt solution, and embedded in paraffin. Sometimes serial sections were prepared of the whole of the lesion, but more often it was found sufficient to examine groups of sections from different levels of the block.

It may be said at once that there is no essential difference between the reaction produced by soluble silica and that which follows the injection of the insoluble forms. The only obvious difference is that the reaction is more rapid and more transient with colloidal silica, and our findings support very strongly the view that  $\text{SiO}_2$  becomes soluble by hydration in the tissue; but, inasmuch as with insoluble silica the reaction is somewhat delayed, the lesions produced by this substance lend themselves rather more readily to analysis than those produced by colloidal silica.

Within three hours after injection of  $\text{SiO}_2$  a recognisable microscopic lesion is produced. It consists of an area of granular coagulation necrosis quite acellular except at the periphery, where a few of the fixed connective-tissue cells of the part can be recognised, and an occasional polynuclear leucocyte.

Five hours after the injection the lesion is more clearly defined. The centre of the focus is still quite acellular, consisting of a coagulum which has entirely replaced the fibrillar connective tissue; with different fixatives the coagulum appears granular to a greater or less degree, but these variations

are entirely mechanical, and the essential process is a coagulation necrosis of the tissue itself, and the serum poured into it. The periphery of the lesion is now more clearly defined by a zone of leucocytes, and by the crowding together of the fixed connective-tissue cells. Beyond the lesion for some distance, even in the neighbouring inguinal lymphatic gland, the capillaries are dilated and filled with blood, and present an active margination and emigration of leucocytes. It may be noted that silica injected into the tissue in this way has no specific action on the endothelium of blood-vessels. Occasionally a venule may be seen containing a delicate coagulum, but for the most part there is no thrombosis. Only in the actual lesion do the venules undergo destruction in common with muscle-fibre, nerves, and the connective tissue.

An hour or two later shows an active invasion of the periphery of the focus by leucocytes, but many of these exhibit degeneration with pyknosis and karyorrhexis of their nuclei. Further, it is obvious that the lesion has not been delimited by the reaction of the tissue, for, especially in the silica lesion, there is a spreading necrosis beyond the marginal zone of leucocytes. In the silicic acid lesion this is not always so noticeable, the explanation being that the deleterious action of the soluble poison is manifested immediately, whereas the insoluble silica becomes soluble gradually, so that its action is potent over a longer period of time.

In seventeen hours the focus is extensive. It exhibits a central acellular zone, varying somewhat in extent, but the greater part of the necrotic area is densely infiltrated with leucocytes, though these themselves are often enough degenerated and fragmented. The coagulum appears to be denser and more inspissated, presumably by the loss of fluid, and it is broken up into spherical masses which are surrounded by leucocytes. The venules are still dilated and engorged.

In forty-two hours the lesion is noticeably more localised. The general characters are the same, but there appears to be some compression of the connective tissue at the periphery, with the formation of a delicate limiting wall. Possibly also actual proliferation of fibroblasts helps in the production of this barrier.

The later stages of the lesion are characterised by the gradual shrinkage of the coagulum, the appearance at the periphery of large mononuclear cells with irregular outlines and vacuolated protoplasm, and the active proliferation of connective tissue with the formation of numerous capillaries. The final stages are those of an organising inflammatory fibrosis, but the process lasts a considerable time, and the remains of the necrotic tissue persist for ten days at least.

These are the general features of the subcutaneous silica lesion as we have observed them. It may be modified in its extent and duration, but such variations as we have observed do not appear to be of fundamental importance. While it is comparatively easy, however, to study the earlier lesions, it is much more difficult in those which have been allowed to go on to three or more days. The macroscopic lesion is itself far from easy to define, and it often happens that the microscopic preparations leave something to be desired. In any series of experiments the later results have been difficult to observe, and this has



necessitated much repetition of the work. It is possible, of course, to define the experimental area by some inert colouring matter such as carbon, but we wished to avoid complicating factors as far as possible, and have therefore relied upon composite pictures obtained from the most satisfactory stages in several experiments. There is, then, a very definite tissue reaction to silica; it is, in fact, so far as our experience of non-bacterial irritants goes, specific. The question therefore arose whether this reaction differed from others in aiding in some specific way the growth of the tubercle bacillus. A comparison between the results obtained by inoculating tubercle bacilli alone and in combination with silica gives interesting results.

#### INOCULATION OF TUBERCLE BACILLI ALONE.

The picture varies enormously according to whether a thick or a thin emulsion of bacilli is used.

With a thick emulsion there is a brisk reaction of the connective tissue, the vessels become dilated, and margination and emigration of leucocytes is obvious as soon as three hours after inoculation. The centre of the lesion consists of a small space in the areolar tissue containing tubercle bacilli. This is surrounded by a thin zone of leucocytes which are actively phagocytosing the bacilli, as are also the fixed connective-tissue cells of the part. Often the wandering phagocytes appear to be emigrating to the lymph-gland, but none can be distinguished within it.

In six hours after inoculation the centre of the lesion consists of an irregular space containing granular material with bacilli at the margin. Immediately around this there is a dense zone of leucocytes, many of them loaded with bacilli. Where clumps of bacilli appear, as often happens with thick emulsions, they are entirely surrounded by leucocytes. Outside this zone the connective tissue is highly cellular, the cells being leucocytes, fixed connective-tissue cells and mast-cells, and there are unusually large numbers of mast-cells around the margin of the lymph-node. Occasionally a stray phagocyte can be seen filled with bacilli, and obviously migrating towards the gland, but it is very rare to encounter such cells actually within the gland.

In nine hours there is a complete alteration in the picture. The general congestion and leucocytic infiltration has passed off, and the loose areolar tissues show little change from the normal. The lesion, however, has become definitely localised as an abscess consisting almost entirely of polynuclear leucocytes. The majority of these contain bacilli, and some are absolutely loaded with them. The general impression given is that of a much larger number of bacilli than in the earlier stages, though they are all intracellular. Around the abscess the connective-tissue fibrils are compressed to form a definite wall, though a delicate one. Outside this are leucocytes in moderate numbers—several packed with bacilli—and there appears to be slight proliferation of the fixed connective-tissue cells.

In twenty-four hours the abscess is even more clearly defined, and contains several small spherical spaces, empty except for bacilli, which are separate, not in clumps. There is intense phagocytosis. Outside the abscess there are a few isolated bacilli and cells which have phagocytosed bacilli, but there is little

general change except for some connective-tissue cell proliferation around the wall.

The later stages of the inoculation with massive doses of tubercle bacilli were studied in preparations from several different experiments, and a fairly satisfactory composite picture of the progress of the lesion has been obtained. The whole process is remarkably quiescent. There is often an indolent proliferation of the fixed connective-tissue elements at the periphery, and the abscess becomes encapsulated with a broad zone of dense fibrous tissue, which is acellular, and appears to be formed partly by compression and partly by the gradual deposit of collagen fibres. Mononuclear cells wander into the abscess in considerable numbers, ranging themselves at the periphery; they are actively phagocytic. In the course of time they replace entirely the leucocytes, which undergo gradual necrosis and lysis, sometimes to such an extent that the centre of the abscess is occupied by a structureless coagulum. In the surrounding areolar tissue occasional polynuclear and mononuclear phagocytes containing many bacilli can be seen. These eventually reach the lymph-gland and deposit their bacilli, which cause a slow, indolent reaction of the endothelial and reticular cells, which form multiple minute microscopic foci in the gland. After a period of three months or more the local lesion consists simply of a group of extremely vacuolated mononuclear cells containing granular bacilli, surrounded by dense fibrous tissue. This nodule is surrounded by a zone of fibroblasts and polyblasts of varying density.

A rather clearer picture of the reaction is obtained by the study of lesions produced by injection of extremely fine emulsions. The following is the protocol of such an experiment:

**24 hours.**—The lesion consists of a very small focus, a cavity in the areolar tissue containing in one or two sections of the series a minute coagulum, around which is a small bunch of cells. Cells are scattered sparsely in the cavity; they are rather thicker at the margin, fading off into the peripheral tissue. There are a few mononuclear elements, fixed connective-tissue cells or polyblasts, but the majority of the cells are polynuclear leucocytes. There are very few bacilli—scarcely one in each section—and they are all phagocytosed.

**2 days.**—A small diffuse focus of cell infiltration consisting of wandering cells, fibroblasts and a few polymorphs. More bacilli can be seen than in the 24-hours lesion, all phagocytosed.

**3 days.**—A very small lesion consisting chiefly of macrophages with foamy cytoplasm; a few bacilli in the cells.

**7 days (Fig. 2).**—A slight indefinite lesion consisting of an accumulation of mononuclear cells, polyblasts and fixed connective-tissue elements. Small groups of bacilli can be seen from time to time lying free in the tissues or within the bodies of fibroblasts. They consist of some ten or more bacilli tightly packed together, and suggest proliferation of the organisms *in situ*. The focus passes into a dilated lymph trunk at the side of the gland, and bacilli can be seen lying free in this with a few mononuclear cells.

The tubercle bacillus possesses only a very slight degree of pathogenicity for the mouse, and this applies equally to human and bovine strains. It can



survive and proliferate in the tissues, but it causes very little reaction. When present in small numbers the local reserves of the tissue appear sufficient to cope with it, though when injected in massive doses it calls forth a pronounced exudation of polynuclear leucocytes. It is readily phagocytosed by the fixed connective-tissue cells or polyblasts as well as by wandering leucocytes, but it can exist for a long period in the bodies of such cells without any apparent change; in fact, it seems likely that it can actually proliferate within phagocytes. It seems to cause no cell necrosis or tissue destruction, and though it may be transported, chiefly by the agency of phagocytes, to lymphatic glands and the viscera, in these secondary foci it gives rise only to a very moderate degree of tissue reaction quite unlike that seen in susceptible animals. It would seem, therefore, that tuberculosis in the mouse ought to provide a particularly favourable opportunity for studying the influence of silica in the tuberculous process.

#### INOCULATION OF TUBERCLE BACILLI WITH SILICA.

We have observed repeatedly that an injection of tubercle bacilli accompanied by silica into the subcutaneous tissue gives rise to a much greater local reaction than an injection of tubercle bacilli alone, and further, that general dissemination is earlier and more active. The silica effect, however, is transient, so that we have, in these experiments, inoculated fresh doses of silica weekly into the local lesion, though the dose of tubercle bacilli has not been repeated. In this way we have produced large abscesses containing enormous numbers of tubercle bacilli, far more than were originally injected, but the common silica lesion produced in this way so complicates the microscopic picture that it is impossible to arrive at a clear conception of what takes place. We have, therefore, resorted to the "early stages" method of analysis. The changes are brought out well in an experiment in which silicic acid was inoculated together with a very fine emulsion of tubercle bacilli suspended in normal saline. In twenty-four hours a considerable lesion is produced, consisting of a central fine coagulum with an outer zone of leucocytes, the whole being bounded by a thin limiting coagulum. A very few bacilli can be seen in the central zone. There is general congestion and leucocytosis in the neighbouring areolar tissue.

In two days the lesion has progressed. The central coagulum is now rather denser and contains rather more bacilli. None of these are as yet phagocytosed. The peripheral leucocytosis is as obvious as in the 1-day preparation.

In three days the central coagulum contains more bacilli and there is now some early reaction in the peripheral connective tissue.

In five days the lesion is more advanced. The central coagulum is dense and contains many more bacilli than in the earlier foci. There is much necrosis of the leucocytes in the intermediate zone, and in the external necrotic zone, which is by now extensive, there is a considerable accumulation of large mononuclear cells with an irregular outline and a vacuolated cytoplasm, probably endothelial cells. There is much new capillary formation at the periphery of the lesion and in the inner part of the external necrotic zone.

The six days' lesion (Fig. 1) is a large one, resembling in general character

the previous ones. At the periphery there is an intense fibroblastic and angioblastic reaction with a zone of condensation of collagen which suggests considerable pressure. The new granulation-tissue contains numerous wandering cells, of which macrophages with vesicular protoplasm form a considerable number. Immediately inside this reactive zone is a band of necrosis containing macrophages and degenerated leucocytes separated from the main central coagulum by a considerable zone of leucocytes, many of them in various stages of degeneration. Macrophages are especially numerous at the margin of the outer coagulated area. In these lesions the bacilli are obviously much increased in number. They can be seen in the outer necrotic zone, usually phagocytosed by macrophages, and in the intermediate leucocytic zone, but they are especially numerous in the central coagulum when they are diffusely scattered in single elements and larger closely intertwined groups.

Seven days: Here the lesion is much smaller, and, in passing, it may be pointed out that the local silica lesion shows invariably this rapid diminution in size, apparently dependent upon loss of fluid immediately after the cessation of the process of progressive tissue destruction. Essentially, however, the lesion is simply a later stages of the previous ones. There is still a central mass of necrotic coagulum, though it is obscured to some extent by leucocytes, and a peripheral necrotic zone with a limiting border of granulation-tissue. The number of mononuclear phagocytes has enormously increased and a considerable proportion of them contain bacilli. The appearances suggest that the bacilli which have been proliferating in the abscess are all engulfed by phagocytes, many of which wander out into the granulation-tissue at the margin of the lesion.

The experiments in which large doses of tubercle bacilli were used in conjunction with silica do not bring out quite so clearly the essential difference between the tubercle silica lesion and the tubercle lesion without silica, inasmuch as such a large number of bacilli is present in both series of lesions. Nevertheless, it is possible to appreciate the development of the bacilli in the necrotic tissue with the formation of large colonies.

#### CONCLUSIONS.

The conclusion we draw from these experiments is briefly as follows: The mouse possesses a high degree of natural immunity to tubercle, and even massive doses of bacilli injected beneath the skin can be tolerated fairly well. In course of time the organisms are encapsuled, and though a certain number of them escape this fate, being conveyed to distant parts by the action of phagocytes, the resulting lesion develops very slowly. With smaller doses of bacilli this tolerance is even more obvious. The isolated bacilli are phagocytosed by leucocytes and also by polyblasts, and in the latter elements they live practically symbiotically, proliferating freely within the cell, which shows no obvious evidence of damage from their presence. When silica is introduced along with the bacilli into the subcutaneous tissues of the mouse there is a very different result. The tissues undergo a considerable degree of necrosis, and though an intense leucocytosis is an important part of the accompanying reaction, the centre of the lesion remains acellular for several days and consists of a structureless coagulum. After a preliminary lag of two or three days



the bacilli proliferate abundantly in this coagulum, protected from the cellular defences of the body. This protection is, however, only temporary, and in a few more days the coagulum is absorbed, and the lesion becomes infiltrated with mononuclear and polynuclear phagocytes, which absorb the bacilli with avidity. In the meantime, however, a small dose of bacilli becomes a dose of considerable magnitude, and, inasmuch as an important factor in determining an infectious process is the number of organisms introduced, a simple explanation is forthcoming of the effect of silica upon such tuberculous lesions as we have described. It is too much, of course, to claim that an exactly similar process takes place in miners' phthisis, but the presence of a definite cell poison must obviously diminish the power of the tissue reaction and so favour the progressive multiplication of the infecting bacilli.

A study of subcutaneous lesions, such as we have described, suggests that the poison does not remain in particulate form at the site of inoculation for any considerable time, but is either fixed locally or is gradually removed by phagocytes and in the tissue fluids. When present it behaves as an active cell poison, but with its gradual disappearance the normal processes of repair become evident; the necrotic tissue is replaced by organising fibrous tissue, and, finally, nothing remains but a dense scar. In the silicotic lung this scarring is the prominent feature, and though tuberculosis and silicosis may co-exist in the same organ, the characteristic fibrosis is often enough the only evidence of previous exposure to silica. It is true that in the midst of the old fibrosed nodules it is possible to distinguish particles of silica, but they are so well encapsuled that it is doubtful whether they can exert any influence on the tissues, since they can scarcely pass into solution. The question therefore arises why, in the absence of tissue necrosis, or with only a minimal amount of cell destruction, the fibrotic lungs are peculiarly liable to tuberculosis? Is the scar-tissue actually less resistant and therefore more vulnerable than normal tissues, or does the successful invasion of the tubercle bacillus depend upon the disorganisation of the circulation of the tissue fluids brought about by lymphatic obstruction?

We have attempted to investigate this point experimentally, but so far our results are not conclusive, and work is still proceeding in this direction. We have found that if subcutaneous injections of small doses of silica are made into the flank of mice at weekly intervals for several weeks an extensive fibrosis is produced. If, now, tubercle bacilli are inoculated into this altered tissue, much more considerable lesions result than in normal control mice. In our experiments these results may have been due to the fact that healing was not complete—that enough necrotic or devitalised tissue was still present to serve as a nidus for the development of the bacilli. But we have found that much the same results, both in regard to fibrosis and in the subsequent reaction to tubercle bacilli, follow the repeated injections of a suspension of carbon. Carbon injected into the subcutaneous tissues of mice remains for the most part *in situ*. It is phagocytosed, but the phagocytes pass away to the neighbouring lymph-nodes only very slowly, and massive doses of carbon give rise, therefore, to blockage of lymphatic vessels and mechanical fibrosis. But though the end-results are similar, the mechanism of the production of the fibrous tissue in the two cases is, of course, entirely different. The silica

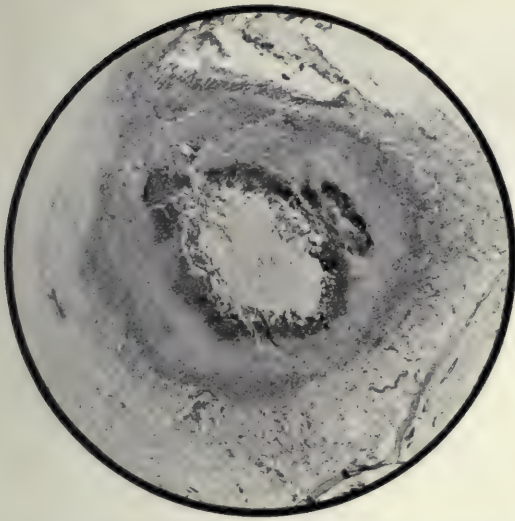


FIG. 1.—Subcutaneous lesion produced in 6 days by inoculation of 2 mgrm. of silica.

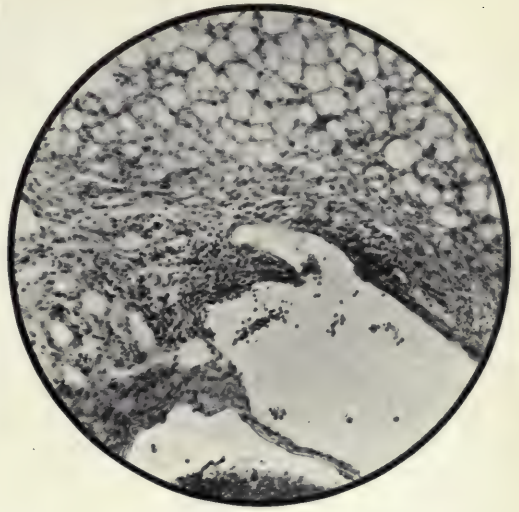


FIG. 2.—Subcutaneous lesion (7 days) following injection of fine emulsion of tubercle bacilli.

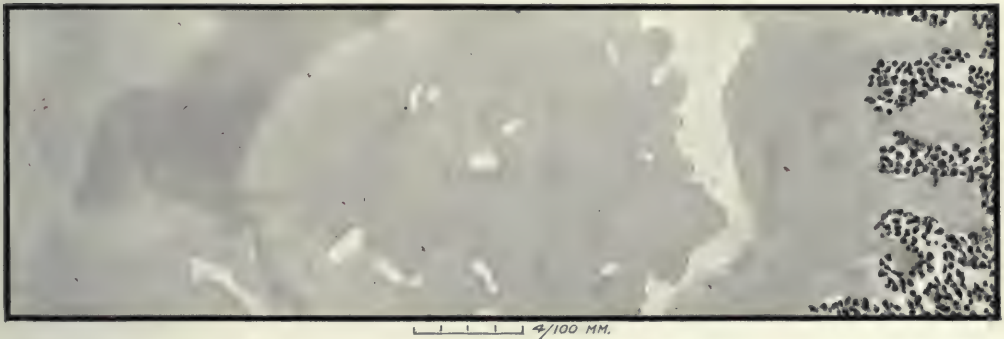


FIG. 3.—Margin of central coagulum in 3 days' silica and tubercle lesion. Early proliferation of bacilli.

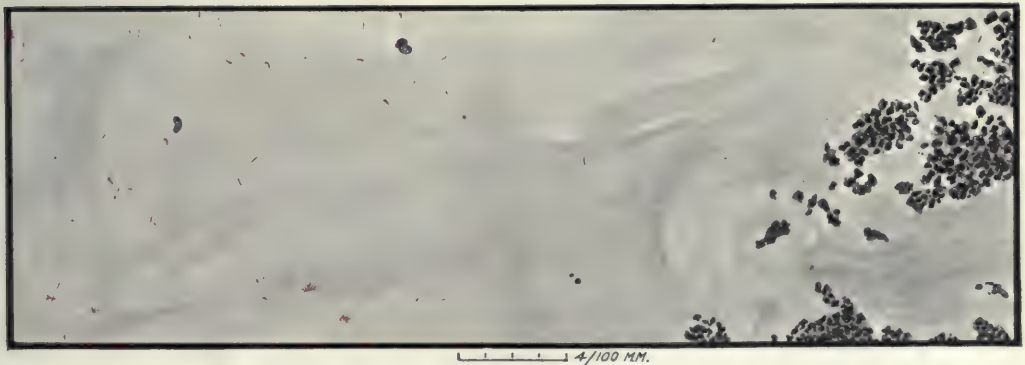


FIG. 4.—Similar lesion, but after 6 days, showing active proliferation of bacilli.





fibrosis is an end-result of a tissue destruction which cannot be produced by carbon.

It is established, of course, that coal miners are no more liable to pulmonary tuberculosis than other members of the general population, and, in point of fact, these results cannot be directly applied to the pulmonary lesions. The experimental conditions are too crude; the sudden injection of considerable localised doses of foreign substances into the tissues at irregular intervals can hardly be compared with the gradual and more or less continuous inhalation of the same substances into the lungs. Moreover, whereas the pulmonary phagocytes can deal adequately with the inert particles of carbon which reach the alveoli of the lungs, the same scavenging mechanism is not available in the subcutaneous tissues. There the carbon tends to remain within the phagocytes which have engulfed it. The phagocytes are anchored locally in considerable numbers, and must eventually die and disintegrate, and in this way sufficient pabulum may be produced to provide favourable conditions for the growth of the bacilli. Further investigations, it is hoped, may throw more light on this aspect of the problem, but at present we are unable to offer any satisfactory solution.

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[The photographs from which figs. 1 and 2 have been made were taken by our colleague, Mr. J. E. Barnard, to whom we express our thanks.]

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## OBSERVATIONS ON A BACTERIOLYTIC SUBSTANCE ("LYSOZYME") FOUND IN SECRETIONS AND TISSUES.

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ONE of us (Fleming, 1922) has noted the occurrence in the tissues and secretions of man and animals, and in some vegetable tissues, of a bacteriolytic substance to which the name "lysozyme" has been applied. The lytic action of this substance was especially manifested on certain non-pathogenic bacteria, although it could also be observed with bacteria which were pathogenic to some of the lower animals or to man. In our experiments we have employed for the most part cultures of a large non-pathogenic coccus (which we have called *M. lysodeikticus*), as this coccus was particularly susceptible to the action of lysozyme, and furnished a convenient indicator for work in connection with its distribution and properties.

*Action of lysozyme on bacteria.*—This question has already been gone into at some length, and it will suffice here to say that if a lysozyme-containing material such as tears be brought into contact with a susceptible microbe such as *M. lysodeikticus*, it exercises a powerful inhibitory, bactericidal and bacteriolytic effect. One of the most remarkable features of the bacteriolytic effect is the rapidity with which it takes place. At a temperature of between 37° and 50° C. an opaque suspension of *M. lysodeikticus* in salt solution subjected to the action of a 1 per cent. solution of tears will be completely cleared in a few seconds. Again, if a small drop of tears be placed on the surface of a well-grown culture plate of this organism, in about one minute there is complete solution of the bacteria, and a clear space marks the position of the tears (Fig. 1). The microscopical appearances observed are illustrated in Fig. 2, which shows the complete disintegration of the cocci in two minutes at room temperature.

*Keeping properties.*—Lysozyme appears to be very stable, and maintains its activity in fluids for many weeks at room temperature. In dried material it does not appear to deteriorate, and it is to be found in very large quantities in commercial dried-egg albumen which had been prepared many months (or possibly even years) before.

*Solubility.*—It is soluble in water or weak saline solutions, but it is absolutely insoluble in alcohol, ether, chloroform, toluol, xylol, or acetone.

*Influence of the salt content of the fluid on the lytic action.*—To 1 c.c. of salt solution of various concentrations was added 0.05 c.c. of a 1 in 10 extract of nasal mucus in water and 0.05 c.c. of a thick suspension of

*M. lysodeikticus*. The tubes were then incubated at 45° C. and lysis noted at various times, with the results given below :

*Lysis observed at times as under :*

Salt concentration.	2 minutes.	10 minutes.	2 hours.	4 hours.
8 per cent. . . . .	0	0	0	0
4    "   . . . . .	0	0	0	±
2    "   . . . . .	0	±	AC	+
1    "   . . . . .	±	+	+	+
0.5   "   . . . . .	+	+	+	+
0 (control) . . . . .	±	+	+	+

+ = Complete.   AC = Almost complete.   ± = Partial.   0 = No lysis.

It is evident therefore that high concentrations of salt inhibit the lysis, and that a concentration of 0.5 per cent. slightly favours it.

*Influence of the reaction of the fluid on lysis.*—To tubes containing 1 c.c. of normal salt solution *plus* acid (HCl) or alkali (NaOH) in varying amounts, was added 0.05 c.c. of a 1 in 2 saline extract of sputum and 0.05 c.c. of a suspension of *M. lysodeikticus*. The lysis noted after incubation is given below:

Concentration of alkali or acid.	Amount of lysis noted at the times indicated.							
	Alkali.				Acid.			
	5 min.	30 min.	2 hrs.	8 hrs.	5 min.	30 min.	2 hrs.	8 hrs.
N/300 . . . . .	0	0	0	0	0	0	0	0
N/900 . . . . .	0	0	0	±	0	0	0	0
N/2700 . . . . .	0	0	0	+	0	0	±	+
N/8100 . . . . .	0	±	+	+	0	±	+	+
N/24,300 . . . . .	0	+	+	+	+	+	+	+
Control, neutral . . . . .	+	+	+	+	+	+	+	+
Control, no sputum . . . . .	0	0	0	0	0	0	0	0

This shows that lysozyme is very susceptible to the reaction of the fluid, acting best in a neutral fluid and being completely or partially inhibited by very small amounts of either acid or alkali.

*Effect of heat on lysozyme.*—Small quantities of saliva were heated to 70° C. for varying times in a water-bath. Of this heated saliva 0.1 c.c. quantities were added to tubes containing 1 c.c. of a suspension of *M. lysodeikticus* and similar quantities of 1 c.c. of a 1 per cent. solution of starch (to make a comparison between the thermal destruction point of lysozyme and ptyalin). The tubes were then incubated for one hour and the lysis of the bacteria or the digestion of the starch noted, with the results given below :

Time during which the saliva had been heated.	Lysis of <i>M. lysodeikticus</i> observed.	Digestion of starch observed.
1 minute . . . . .	±	Digested.
2 minutes . . . . .	±	"
3    "   . . . . .	±	"
5    "   . . . . .	±	"
7½   "   . . . . .	0	Not digested.
Unheated control . . . . .	+	Digested.



This shows that in saliva lysozyme is destroyed by heat at 70° C., and that the rate of destruction is comparable with that of the diastatic ferment ptyalin.

In sputum, nasal mucus and some of the solid tissues it was found that heating for 10 minutes to 75° C. sufficed to destroy the lytic and inhibitory power.

Fig. 3 illustrates the destruction of the inhibitory power of cartilage by heat at 75° C.

In tears a higher temperature was required for complete destruction of the lysozyme. Heating to 72° C. for 10 minutes reduced the titre to one-half and 75° for the same time to one-quarter of the original, but it required boiling for over 30 minutes to completely destroy it.

*Influence of temperature on the velocity of the lysis.*—Lysis occurred slowly in the ice chest, so that although with 1 in 100 tears there was complete lysis of a suspension of *M. lysodeikticus*, there was no visible lysis with 1 in 1000 tears in 2 hours, whereas at 37° C. a 1 in 135,000 dilution of the same sample of tears showed complete lysis in that time. The rate of lysis is increased with the temperature up to about 60° C. After 24 hours, however, the titre is about the same, whether the temperature be that of the room, 37°, or 50° C.

*Influence of certain organic substances on lysozyme.*—It was found that substances such as alcohol or acetone precipitated the whole of the lysozyme from an albuminous fluid, but they did not destroy it, since if the precipitate was collected and placed in normal salt solution, it gave up to the fluid a strong bacteriolytic substance. The action of other fluids on lysozyme was investigated especially with a view to the preparation of sterile human secretions and tissues for experimental purposes.

Pus was taken from an empyema, and to quantities of about 5 c.c. an excess of toluol, thymol or chloroform was added. The tubes were tightly corked and shaken at intervals for 24 hours, when portions were removed, centrifuged, and the supernatant fluid titrated for lytic substance.

Four months later these same fluids were again titrated, having in the meantime stood on the bench in tightly corked tubes. The following results were obtained :

Preservative used.	Bacteriolytic titre after 2 hours' incubation at 45° C.									
	After 24 hours.					After 4 months.				
	1 in					1 in				
	100	200	400	800	1600	100	200	400	800	1600
Toluol . . .	+	AC	...	Trace	0	+	AC	±	Trace	0
Thymol . . .	+	+	±	„	0	Trace	0	0	0	0
Chloroform . .	+	+	±	„	0	+	AC	±	Trace	0
Control (pus without addition)	+	AC	±	„	0	+	AC	±	„	0

It will be seen that toluol and chloroform had no action on the lysozyme even after 4 months. Saturated thymol, on the other hand, had a deleterious effect.

The action of various fluids on the inhibitory action was investigated in

another way. Fishes' eggs (pike) were found to contain lysozyme, and it was shown that such lysozyme was essentially the same as that contained in human tissues. When these eggs were embedded in an agar plate, the surface of which was subsequently planted with *M. lysodeikticus*, there was around each egg a clear area where the growth was inhibited, and each of these areas was approximately the same size. These eggs then furnished an easy means for the investigation of the action of volatile fluids on lysozyme. It was first of all determined that a short exposure to acetone (24 hours) was without effect; then the eggs were placed in excess of acetone for purposes of dehydration. After 24 hours they were removed, dried, and small quantities were placed in various fluids and allowed to remain for 3 months, the tubes being shaken at intervals. They were then removed, thoroughly dried and imbedded in an agar plate along with some of the original eggs, which had merely had a short dehydration with acetone and which had been afterwards kept in a dry condition. The plate was planted with *M. lysodeikticus* and incubated and the result is shown in Fig. 4, from which it will be seen that the inhibitory power has not been destroyed by three months' treatment with chloroform, ether, xylol, toluol, alcohol, or acetone.

*Filtration of lysozyme.*—If a lysozyme-containing fluid be passed through a filter of porcelain, filter-paper, or cotton-wool, a large amount of the lytic substance is at first absorbed by the filter, so that it is only by filtering large quantities of material that any of the lysozyme passes through.

Lysozyme is also absorbed by substances such as charcoal added to the fluid.

*Comparison of the lytic action on dead and living microbes.*—Two identical sets of serial dilutions of tears were made. To one set was added a small quantity of a suspension of a young live culture, and to the other the same quantity of the same suspension which had been boiled for 5 minutes. The tubes were then incubated at 45° C. and it was found that lysis proceeded at the same pace in each set. There was a difference, however, in that the tubes containing the boiled microbes never became absolutely clear even after long incubation in contact with lysozyme; there always remained a faint opacity, but this opacity rapidly cleared up when a small quantity of trypsin was added, showing that it was due to a small amount of proteid matter in the bacterial suspension, which had coagulated in the process of boiling.

It appears, therefore, that the lytic action is manifested equally on living and dead organisms.

*Distribution of lysozyme to M. lysodeikticus in the human body.*—It has been shown (Fleming, 1922) that in all the tissues and in all the secretions and excretions tested, with the exception of cerebro-spinal fluid, sweat and urine, there exists lysozyme to *M. lysodeikticus*. To these exceptions might be added normal faeces.

It might be of interest to compare quantitatively the lytic power of body fluids and some of the tissues. The fluids were tested at various times as they became available by making serial dilutions in quantities of 1 c.c., and to each dilution adding one drop of a suspension of *M. lysodeikticus*. The comparison is not absolutely accurate in that the bacterial suspension was not always of the same strength, but the figures are sufficiently close as to give a



very fair indication of the lysozyme content. The results obtained with normal and pathological fluids are given in the following table:

*Lytic Power of Normal and Pathological Fluids.*

Fluid.	Highest dilution giving complete lysis.	Lowest division giving no lysis.	Time of incubation.
Cerebro-spinal fluid . . . . .	—	1 in 1	Up to 24 hours.
Normal urine . . . . .	—	1 in 1	„
Sweat . . . . .	—	1 in 10	„
Blood—			
{ Citrated plasma . . . . .	1 in 80	1 in 640	1 hour.
{ Serum from defibrinated blood . . . . .	1 in 80	1 in 640	„
{ Serum off blood-clot . . . . .	1 in 40	1 in 320	„
*Blood-serum . . . . .	1 in 270	1 in 3420	„
*Tears . . . . .	1 in 40,000	1 in 325,000	„
*Saliva . . . . .	1 in 300	1 in 2700	„
*Sputum . . . . .	1 in 13,500	1 in 121,500	„
*Nasal mucus . . . . .	1 in 13,500	1 in 121,500	„
Pleural effusion (clear) . . . . .	1 in 81	1 in 2187	„
Ascitic fluid . . . . .	1 in 50	1 in 1600	„
Hydrocele fluid . . . . .	1 in 18	1 in 1438	„
Pus (1) . . . . .	1 in 700	1 in 20,000	„
(2) . . . . .	1 in 100	1 in 1600	2 hours.
Urine containing pus and albumen . . . . .	1 in 32	1 in 512	1 hour.
Ovarian cyst fluid . . . . .	1 in 10	1 in 160	„
Fluid from parotid cyst . . . . .	1 in 100	1 in 3200	„
Semen . . . . .	1 in 20	gave partial lysis after 3 hours.	

In connection with blood it is interesting to note that fibrin contains a considerable amount of lytic substance, and that citrated plasma and the serum from defibrinated blood both give a higher titre than does serum which has stood for any length of time on the clot. The fibrin appears to abstract from the serum a certain amount of its lytic substance, and it may well be

\* From the same individual tested at the same time.

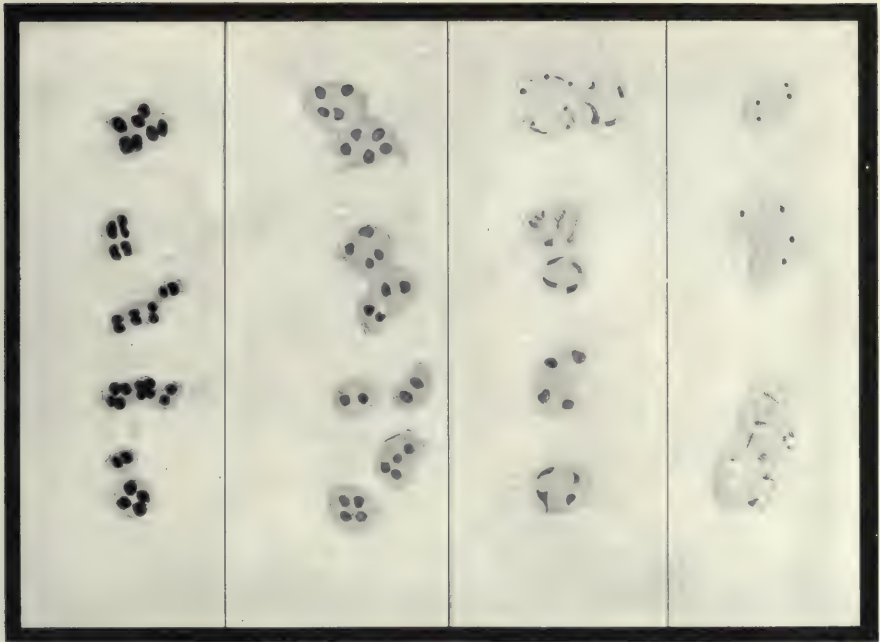
DESCRIPTION OF PLATE.

FIG. 1.—Photograph of a plate culture of *M. lysodeikticus* on which had been placed a small quantity of tears in the form of the letter *T*. Note complete clearing of that portion of the culture on which the tears had been superposed.

FIG. 2.—Microscopical appearances observed during the solution of *M. lysodeikticus* by tears. (Hiss. capsule stain.) 1. Before being acted on. 2. After 10 seconds' contact with tears 1/4 at room temperature. 3. After 30 seconds' contact. 4. After 2 minutes' contact.

FIG. 3.—Photograph of a plate showing the destruction of the inhibitory power of cartilage by 10 minutes' heating at 75° C. L. Unheated. R. Heated.

FIG. 4.—Photograph of a plate showing the inhibitory effect of pike's eggs which had been exposed to the action of various fluids for a period of 3 months.



1.

2.

3.

4.

FIG. 2.

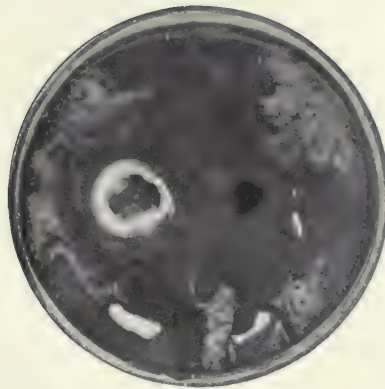


FIG. 3.



FIG. 1.

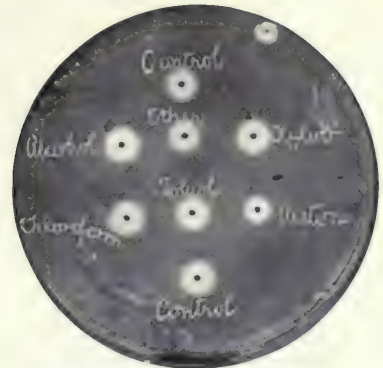


FIG. 4.





that this lytic power of fibrin is of importance in connection with the death of bacteria in a blood-clot.

We have investigated the question of whether the amount of lytic substance was constant in the blood-serum or tears of different individuals. Tears and blood-serum were collected from 6 laboratory workers and were titrated for lytic power with the following results:

Individual.	Lysis observed after 1 hour's incubation at 45° C.													
	Tears.								Serum.					
	Dilution (in thousands).								Dilution.					
	3	9	27	81	243	729	2187	10	30	90	270	810	2430	
V. D. A—	+	+	AC	±	±	Trace	0	+	+	AC	±	Trace	0	
A. F—	+	+	AC	±	Trace	Trace	0	+	AC	±	Trace	0	0	
F. M—	+	+	±	Trace	Trace	0	0	+	+	±	Trace	Trace	0	
K. N—	+	+	AC	±	Trace	Trace	0	+	AC	±	Trace	0	0	
A. F. H—	+	AC	±	Trace	0	0	0	+	+	±	Trace	0	0	
E. J. S—	+	AC	±	±	Trace	0	0	+	+	AC	±	Trace	0	

From this it will be seen that there were definite differences between the different individuals.

It will be noticed that the titres obtained in this experiment are comparatively low. This apparently was due to the strain of coccus used being less sensitive than normal to the lysozyme. The strain used in this case had not been subcultured for two months.

The lysozyme content of certain tissues was investigated by placing the minced tissue in an excess of acetone for 24 hours for dehydration, drying the tissue, and then grinding up 1 gm. of the dry tissue with 20 c.c. of normal salt solution and allowing it to extract for 24 hours. The extract was centrifuged and the clear supernatant fluid was titrated for lytic power.\* The results obtained were as follows:

Tissue.	Highest dilution giving complete lysis.	Lowest dilution showing no lysis.
Liver . . . . .	1 in 400 (almost complete)	1 in 3200
Tonsil . . . . .	1 in 400	1 in 6400
Kidney . . . . .	1 in 100	1 in 800
Intestine . . . . .	1 in 500	1 in 8000
Stomach . . . . .	1 in 1000	1 in 32,000
Meninges . . . . .	1 in 400 (almost complete)	1 in 3200
Skin . . . . .	Less than 1 in 100	1 in 800
Cartilage . . . . .	1 in 1300†	—
Turnip . . . . .	1 in 4	1 in 32

It will be seen that of all the tissues tested cartilage is much the most powerful as regards lytic power. The cartilage tested in this case was derived

\* This method of investigating the lytic power of tissues is almost identical with that described by Turro (1921) in his work on the bacteriolytic power of leucocyte and tissue extracts. This work was unknown to us when our experiments were carried out.

† After 5 minutes' incubation. The other results are after 1 hour's incubation.



from the articular cartilage of the patella. It may be noted that meninges possessed a considerable amount of substance, but it was found that brain tissue was very weak in this respect, although accurate titrations could not be made owing to the fact that the extracts were so opaque that bacteriolysis could not be readily observed.

In another communication (Fleming and Allison) we have shown that when bacteria are grown in the presence of a lysozyme-containing material there is at first complete inhibition of growth, but later a few scattered colonies appear, and these colonies are resistant to the lytic action of the lysozyme. Not only are they resistant to the lytic action of the tissue or secretion in contact with which they originally grew, but they are equally resistant to the lytic action of all other tissues. For instance, it was found that a strain of *M. lysodeikticus* grown in contact with nasal mucus was not more resistant to the lysozyme of nasal mucus than it was to that of liver, egg-white or turnip. It appears, then, that the lytic substance is the same in whatever tissue it occurs.

We have also shown that after solution of a large number of cocci there is an increase in the lytic content of the fluid, and that this increase in the lytic substance is dependent on the number of microbes dissolved. This is clearly shown in the following experiment:

To 2 c.c. quantities of tears (1 in 100) varying amounts of *M. lysodeikticus* were added. Incubation was carried out for 15 minutes at 45° C., when lysis was complete. The contents of the tubes were then titrated for lytic power towards *M. lysodeikticus* with the following results:

Number of cocci previously dissolved by tears.	Lysis observed after 1 hour's incubation. Dilution of tears (in thousands).						
	5	10	20	40	80	160	320
0 (control)	+	AC	±	0	0	0	0
30 million per c.c.	+	AC	±	Trace	0	0	0
300     "     "	+	+	AC	AC	±	0	0
3000   "     "	+	+	+	AC	±	0	0
30,000   "     "	+	+	+	AC	±	Trace	0

It appears, therefore, that the lysozyme acts on the bacteria in such a way that there is liberated from them a lytic substance to the same strain of bacterium.

*The relation of lysozyme to "bacteriophage."*—This increase in the lytic power after solution of large numbers of microbes resembles to some extent the phenomenon of the "bacteriophage," and it may be of interest here to contrast the properties of lysozyme with those of the "bacteriophage." d'Hérelle maintains that the bacteriophage is a living ultramicroscopic organism acting as a parasite on the bacteria, but such an explanation of lysozyme action would be impossible. There are variations also between the properties of the two substances which make it clear that they are essentially different. The action of the bacteriophage is relatively slow and is only manifested on young active bacterial cultures, whereas lysozyme action is very rapid and the lysis takes place with dead as well as with living bacteria. Again, the range of temperature within which the bacteriophage will act is a compara-

tively narrow one, whereas lysozyme action will take place between 4° C. and 65° C. The greatest difference, however, is that with lysozyme it has been found impossible to transmit the lytic principle in series as can be done with the bacteriophage, and that in solid cultures of bacteria *plus* lysozyme there are never seen the small round clear areas which are characteristic of the bacteriophage.

It has been mentioned above that Turro obtained in tissue extracts a bacteriolytic substance, but he does not enter sufficiently into the details of the properties of the lytic substance to say whether or not it is the same as the substance we have described under the name "lysozyme." He states, however, that the lytic principle in leucocytes was not stable, so that the extracts or even the solid substance may become inert in a few days. We have shown that lysozyme is very stable, and from the table on p. 254 it will be seen that an extract of leucocytes retains its power for at least four months.

Gengou (1921), also, has described a bacteriolytic substance which he has found in extracts of leucocytes. He found, however, that the lysin was absorbed by saturation with the microbe in contrast to the increase in the lytic substance which we have described above.

#### CONCLUSIONS.

There exists in human secretions and tissues (with few exceptions), as well as in animal and some vegetable tissues, a bacteriolytic substance which has properties similar to those of ferments. This lytic substance is especially active towards some non-pathogenic bacteria (and in all probability it is the cause of such bacteria being non-pathogenic), but it is active also on certain pathogenic species. So far as the indicator microbe (*M. lysodeikticus*) is concerned lysozyme is present in greatest amounts in such secretions as tears, nasal mucus, and sputum; in the tissues, especially in cartilage; while it is present in very large amount in egg-white. It may be noted that it is in just such situations that protection from bacterial contamination is required. The conjunctiva is constantly coming into contact with air-borne bacteria, as also is the nasal mucous membrane and the lining of the air-passages, and it is in the secretions covering these membranes that the lytic substance is present in greatest abundance. Cartilage also is lacking in the protection which normally comes from an abundant blood supply, while egg-white has little protection from contamination other than its inherent anti-bacterial power.

The lytic substance, lysozyme, is very stable, retaining its activity in a fluid medium at room temperature for several months. It acts equally on dead and living microbes. If small quantities are filtered the whole of the lysozyme is retained on the filter, but after the filter becomes saturated it readily passes through. Its action is manifest at temperatures between 4° C. and 65° C., but it is slower at the lower temperatures. It is not destroyed by long contact with the common organic solvents.

Strains of bacteria resistant to lysozyme are readily developed, and it is found that whatever the source of the lysozyme to which the bacteria are made resistant, they are equally resistant to the lytic action of all secretions and tissues, animal or vegetable, thus indicating the essential similarity of lysozyme from different sources. The lytic substance is not absorbed by



saturation with the indicator coccus, but, on the other hand, is increased, and the amount of the increase is dependent on the number of cocci dissolved. This increase in the lytic content is reminiscent of the "bacteriophage," but a comparison of the properties of this with lysozyme brings out some fundamental differences.

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## AGGLUTINATION OF WASHED RED BLOOD-CORPUSCLES BY COLLOIDAL SILICA.

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DURING some preliminary experiments to ascertain the effects of colloidal silica upon phagocytosis of tubercle bacilli it was noticed that, in opsonic mixtures containing diluted blood serum, there was immediate agglutination of the red corpuscles when the washed blood suspension was added, although there seemed to be no such agglutination in preparations where the serum was more concentrated. Since the serum under examination was from the same source as the washed blood-cells, it looked, at first sight, as if the presence of the silica had brought to light some kind of paradoxical iso-agglutination of the erythrocytes, but a moment's reflection showed that the facts might be equally well explained on the assumption that the colloidal silica had some property analogous to that described for "ox colloid" by Bordet and Gay (1906). But if this were the case, it remained to be explained why there was no visible flocculation in the tubes containing the more concentrated serum. As this seemed to point rather to an interference with the agglutination by strong serum than to an agglutinating action of the serum when diluted, it was clearly necessary to ascertain whether the phenomenon could be reproduced with silica and salt solution in the absence of serum.

To settle this point, the following experiment was carried out:

*Experiment I.*—To a Dreyer agglutination tube, the following reagents were added:

- 1 vol. colloidal silica.
- 1 vol. of 1·5 per cent. salt solution.
- 2 vols. of normal salt solution (to dilute).

To this mixture was added a drop of suspension of washed blood-corpuscles.

The corpuscles were at once flocculated, this effect being well brought out by blowing a portion of the mixture on to a glass slide as in blood typing. The flocculi were large and the drop of agglutinated blood presented a sharp contrast to a "control" preparation made up with the same cells and normal salt solution.

This experiment served to show that the agglutination was quite independent of the presence of serum, thus setting aside all possibility of iso-agglutination and further proving that the previous sensitisation of the red cells assumed to be necessary in the "conglutinin" phenomenon of Bordet and Gay is not needed for this silica agglutination of red corpuscles.

It is clear that the clumping is due entirely to physical properties in the mixture of cells, salt solution and colloidal silica and that the so-called "immune substances" play no part.

How, then, was the apparent inhibition of the agglutination by the higher concentrations of serum to be explained? During the first steps in the opsonic experiment to which reference has been made above, the fact had been noted that the admixture of silica colloid and human serum led, in the higher concentrations of the latter, to a marked opacity in the fluid. This opacity appeared to be accompanied by an increase in viscosity, the mixture of silica colloid and concentrated serum seeming to result in a gel. It occurred to the author that this state of gel might impede the flocculation of the red cells by mechanical obstruction and that this might be the explanation of the absence of "clumping" of the red cells with the concentrated serum. To test this assumption, the following method was tried:

*Experiment II.*—Three Dreyer tubes were labelled (1), (2), and (3).

To tube (1) were added:

- 1 vol. normal salt solution.
- 1 vol. serum (C.M.A.).
- 1 vol. of mixture of equal parts silica colloid and 1.5 per cent. salt solution.
- 1 vol. washed red blood-cells.

Result: No visible clumping or sedimentation of red cells.

To tube (2) were added:

- 2 vols. normal salt solution.
- 1 vol. mixed silica colloid and 1.5 per cent. salt solution.
- 1 vol. of washed red blood-cells.

Result: Immediate and gross clumping of the red cells.

To tube (3) were added:

- 1 vol. normal salt solution.
- 1 vol. mixed silica colloid and 1.5 per cent. salt solution.
- 1 vol. washed red blood-cells.

Result: Immediate and gross clumping of the red cells.

As soon as this "clumping" had become marked, 1 volume of serum (C.M.A.) was added to tube (3) and the whole thoroughly mixed by blowing bubbles of air through the fluid from a capillary pipette. This served to disperse the clumps and these now showed no tendency to re-flocculate or sediment, the appearance of tube (3) being almost identical with that of



tube (1). Examination of tubes (1) and (3) with a hand lens showed that, although no sedimentation was taking place, there was a decided tendency to minute clumping of the red cells.

From this experiment it is probable that the absence of marked clumping and agglutination in the presence of concentrated serum is due to the formation of a gel of sufficient firmness to mechanically hold the clumps from flocculating and sinking in the fluid.

To what extent does dilution of the silica colloid affect its power of clumping red blood-cells?

*Experiment III.*—A mixture of equal parts of silica colloid and 1.5 per cent. salt solution was prepared and from this dilutions were made in normal saline as follows:  $\frac{1}{2}$ ,  $\frac{1}{4}$ ,  $\frac{1}{8}$  and so on up to  $\frac{1}{512}$ . To each tube was now added a measured volume of red blood-cells. Within five minutes at room temperature there was complete sedimentation in all the tubes up to  $\frac{1}{64}$ , while there was clumping, though with smaller flocculi and less sedimentation, up to  $\frac{1}{512}$ . A further series of dilutions to  $\frac{1}{5000}$  were now prepared. In these there was no visible agglutination in 15 minutes but all showed traces of clumping after one hour.

#### CONCLUSIONS.

Colloidal silica solution to which sufficient salt has been added to bring the mixture up to 0.75 per cent. of NaCl brings about rapid agglutination of washed red blood-corpuscles and this result can still be obtained after considerable dilution.

The flocculation and sedimentation of washed red cells by colloidal silica as above is to a great extent prevented by the presence of concentrated human serum, this inhibition disappearing as the serum is made more dilute. The inhibition seems to depend upon the formation of a gel.

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## A NEW MEDIUM FOR THE ISOLATION OF *B. DIPHTHERIÆ*.

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THE reasons which led to this medium being employed were as follows: The work initiated by Wright (1915), and amplified by Flemming, Colebrook and myself (1920), in which it was shown that the complete or partial neutralisation of the anti-tryptic power enormously increased the value of serum as a medium, suggested that if a suitable proportion of such a trypsin and serum mixture was added to agar the growth of *B. diphtheriæ* might be greatly increased. It was also thought that these conditions were in all probability those that obtained in the false membrane where the bacteria grew so freely. Preliminary trials were therefore made in which the growth of *B. diphtheriæ* on media prepared by adding varying percentages of trypsinised serum to agar was compared with that on agar to which a corresponding amount of untreated serum had been added, with the result that media containing trypsinised serum showed itself in every way superior. This medium had, however, two disadvantages: (1) It was difficult to recognise the colonies of *B. diphtheriæ* from those of other organisms; (2) many of the organisms commonly associated with *B. diphtheriæ* in throat swabs also grew very luxuriantly. Following the work of Conradi and Troch (1912), and J. F. Smith (1914), it was decided to add a percentage of potassium tellurite in hopes of checking the growth of the associated organisms and also of giving a distinctive appearance to the colonies of *B. diphtheriæ*.

### PREPARATION OF THE MEDIUM.

Reagents required: (a) Nutrient agar; (b) solution of potassium tellurite 1 per cent.; (c) sterile trypsinised serum.

(a) The nutrient agar preferably employed is one made with broth prepared by digesting meat with trypsin, as described by me (1914), but ordinary agar made with peptone broth gives almost identical results. Two per cent. of agar should be dissolved in the broth.

(b) The solution of potassium tellurite is somewhat troublesome to prepare, as a proportion of the salt usually fails to dissolve. This difficulty can be



surmounted by dissolving as much as possible of the salt in a proportion of the distilled water, allowing the insoluble portion to settle, and then pouring off the clear, supernatant layer as completely as possible. The deposit is rubbed up with a solution of 10 per cent. potassium hydrate, added drop by drop, until completely dissolved. In order to neutralise the excess of alkali, hydrochloric acid is cautiously added until a precipitate commences to appear. This fraction is then added to the portion of the salt which readily dissolved, and the solution is made up to the requisite volume with distilled water. Once dissolved the solution remains clear for a considerable period, if kept in a vessel securely corked.

(c) Sterile trypsinised serum. This consists of serum to which sufficient trypsin has been added to neutralise partially the antitryptic power.

It may be prepared either (1) by adding a sterile sample of trypsin to sterile serum, or (2) by sterilising the mixture of serum and trypsin by filtration.

(1) Sterile serum—horse-serum is usually employed—is procured either by bleeding the animal, with aseptic precautions, or by adding 0.25 per cent. of chloroform to serum obtained from the slaughter-house and carefully freed from red blood-corpuscles. This mixture is then placed in a tightly glass-stoppered bottle and kept at a moderately high temperature, such as 37° C., or better 42° C., for a few days. Sterile glycerinated solutions of trypsin may be obtained in sealed glass ampoules either from Messrs. Fairchild & Co., or from the Digestive Ferments Co., U.S.A. Both these products possess a similar tryptic power, and appear reliable as regards sterility.

To neutralise the antitryptic power of 100 c.c. serum from a normal horse rather more than 5 c.c. of either of these products is necessary, but for the present purpose the addition of 2 to 4 c.c. of the sterile trypsin to 100 c.c. of the sterile serum is sufficient. Such a mixture keeps for months, and is best stored in quantities sufficient for making up a single batch of medium.

An easy method of testing the strength of a trypsin solution is as follows: Fresh milk is taken and the cream removed by centrifuging. Sufficient calcium chloride solution is then added to bring the concentration up to 1 per cent.

Graded dilutions of the trypsin are then made, and an equal volume of each dilution and of the milk are mixed together in a series of dwarf test-tubes. The tubes are placed in a water-bath at 50° C. for 15 or 30 minutes, after which they are examined to ascertain the highest dilution of the trypsin which causes clotting of the milk. This method is founded on the observation of Mellanby and Woolley (1912-13), that trypsin caused the clotting of milk to which calcium chloride had been added.

When it is desired to ascertain the antitryptic power of a serum, the following mixtures are made in a series of dwarf test-tubes: one volume of milk containing 1 per cent. of calcium chloride; one volume of serum; one volume of graded dilutions of trypsin. These tubes are placed in a bath at 50° C. for 15 or 30 minutes and then examined for the presence of clot. By noting the first tube of the series which shows absence of clotting, it is a simple matter to calculate the amount of the solution of trypsin necessary to neutralise the antitryptic power of a known volume of serum. This method has been fully described by Wright and Colebrook (1921).

(2) When sterile serum is not available, or in making large quantities, serum is obtained from blood procured from the slaughter-house, and to this is

added sufficient of any of the commercial glycerinated solutions of trypsin which are sold in bulk. Messrs. Allen & Hanburys, for instance, supply a suitable preparation under the name of *Liq. trypsinæ co.* Of this product 5 to 8 c.c. should be mixed with every 100 c.c. of horse-serum, as the trypsin content is not so high as that sent out in ampoules. The resulting mixture is then filtered through a porcelain candle of medium porosity and distributed with strict aseptic precautions into sterile vessels of suitable capacity.

Having obtained the reagents, the medium is prepared as follows: The agar is melted; and to every 100 c.c., 4 c.c. of the 1 per cent. potassium tellurite solution is added; this mixture is then cooled to between 65° and 60° C., and 15 c.c. of the trypsinised serum poured in. After thorough mixing, the medium is distributed into sterile tubes with precautions against contamination similar to those employed in making blood or serum agar, and at once sloped. The medium keeps well. The above proportion of trypsinised serum gives the most constant results, but less—for instance, 10 c.c. to every 100 c.c. of agar employed—is sufficient for all practical purposes.

#### THE APPEARANCE OF THE GROWTH RESULTING FROM IMPLANTATIONS FROM THROAT SWABS.

As the medium is perfectly transparent, and as colonies of the *B. diphtheriæ* and some organisms of the same group can be recognised with considerable certainty by inspection, it is advantageous to implant the surface somewhat sparsely so that individual colonies may have space to develop, and may be easily picked off for isolation or microscopical examination. A convenient practise is to draw the charged swab up the centre of the agar surface and then to distribute the material deposited over the whole surface by close strokes of a platinum loop, made at right angles to the track of the swab. After 18 to 24 hours' incubation, if *B. diphtheriæ* or certain other organisms of this group be present, colonies having the following characters will be apparent: in size they vary from about 0.75 to 1 mm. or more in diameter; in shape they resemble a somewhat flattened hemisphere with regular outline; in structure they are remarkably granular—a feature best made out with the aid of a hand lens; in colour, which is the most striking character, the centre is a dusky grey-black, while the peripheral portions are a somewhat opalescent greyish white, the whiteness being accentuated by the granular structure.

Colonies of Hofmann's bacillus have almost identical characters; the blackish colour of the centre is, however, usually less intense. *B. xerosis* and some other members of the diphtheroid group give colonies which are similar in every way to those of *B. diphtheriæ*.

Colonies of streptococci, which are usually the most abundant associated organisms, are very small, flat, and so translucent as to be visible only on close inspection. They have no trace of black colour at this stage of development. Only one organism has been experienced which at this stage of growth gives a colony in any way comparing in size and appearance with those of *B. diphtheriæ*. This is a staphylococcus which occurs in a small percentage of throat swabs. The colonies, however, differ from those of the diphtheroid group, mentioned above, in that the black colour is much more intense and more diffused throughout the colony. The granular structure, which is such a marked feature of colonies



of *B. diphtheria*, is replaced by a homogeneous smoothness—in fact, the colony resembles very closely a spot of wet black paint.

After incubation for 48 hours, the colonies of *B. diphtheria* have increased in size so that they may measure 2 or more millimetres in diameter. The black colour is much accentuated, and has spread throughout the whole colony. The central portion is usually somewhat thickened, the edges are smooth and regular; the general appearance at this and later stages of incubation may be compared to a flattened drop of partially dried black paint.

Colonies of streptococci have almost the same appearance as after 24 hours' incubation and usually remain inconspicuous. When examined carefully, however, a minute black spot may be made out in the centre of the colony, and this tends to become more marked after prolonged incubation.

From this description it is apparent that *B. diphtheria* and some other members of this group give colonies, after 24 hours' incubation, of a distinctive type, the principal features being their large size, granular structure, grey-black centre, and greyish-white peripheral portion. After 48 hours' incubation the colonies resemble a flattened drop of partially dried black paint. If no such colonies can be seen after 48 hours, the culture may be safely discarded as a negative result as regards the presence of *B. diphtheria*. Much greater caution is necessary in deciding by inspection that *B. diphtheria* is absent after 20 to 24 hours, as, unless the colonies are well separated, the distinctive characters may not have developed, and it is always advisable to make a microscopical preparation from any portion of the surface on which the growth is abundant.

When the colonies of staphylococcus are present, although with but little practice they can be distinguished with moderate certainty from those of the diphtheria group, microscopical examination is always necessary.

If isolation is desired, a convenient method is to draw out the end of a capillary pipette to a hair-fine tube in the peep flame of a Bunsen burner. This is then broken so that a portion of the hair-fine tube about 1 cm. long is left at the end. The suspicious colony is then touched with the point of the hair-fine tube. The pipette is then withdrawn and a single streak made up the centre of a fresh tube of medium. This deposits on the surface sufficient organisms to give an abundant culture, and separate colonies are assured by distributing these by close strokes of a platinum loop made at right angles to the central streak. The inside of the hair-fine tube will, on examination, be found to contain amply sufficient organisms to make a microscopical preparation, a plug of organisms, often half to one millimetre in length, having been drawn up by capillary attraction into the lumen. These can be easily washed out by placing a small drop of water on a slide, which is then sucked into and blown out of the capillary tube.

The microscopical appearance of *B. diphtheria* grown on this medium differs only from those grown on Loeffler's medium in that the metachromatic granules are not quite so abundant; they are, however, sufficiently numerous to make the use of the diagnostic stains based upon their presence perfectly satisfactory.

Virulence of emulsions of *B. diphtheria* was, in the case of nineteen strains investigated, similar in every way to that of emulsions made from cultures grown on Loeffler's medium.

## RESULTS OBTAINED FROM THIS MEDIUM IN COMPARISON WITH THOSE FROM LOEFFLER'S MEDIUM.

In order to obtain a reliable result, the following technique was adopted :

Swabs were obtained from a number of cases of diphtheria admitted to the North-Western Fever Hospital, through the agency of Dr. Mair, to whom I now wish to convey my thanks. These cases were in all stages of the disease, and many of them had received antitoxin. On receipt of the swab the first process was to moisten it thoroughly in a tube of sterile broth ; the second was to draw the swab up the centre of a tube of Loeffler's coagulated serum, rolling the swab during this process so that all aspects of the cotton-wool were brought into contact with the surface of the medium ; the third was to carry out a similar implantation on to a tube of trypsinised serum agar ; the fourth was to implant a tube of broth containing similar percentages of trypsinised serum and potassium tellurite solution. This last method was latterly discarded as unnecessary, as although organisms microscopically resembling *B. diphtheriæ* developed in all the broth tubes planted from swabs which gave colonies of this bacillus, such organisms were never found in the broth tubes when no colonies were seen on the solid medium.

From the results given in the table it will be seen that swabs from 29 cases were examined. *B. diphtheriæ* was found and isolated from the primary cultures from 19 of these by means of the trypsinised serum tellurite medium, while from Loeffler's medium *B. diphtheriæ* was microscopically recognised on 16 occasions, but isolation from primary cultures almost always failed. In no case was *B. diphtheriæ* found in the cultures on Loeffler's medium when absent from those on the trypsinised serum agar.

Medium employed.	No. of swabs examined.	No. of positive results.	Percentage of positive results.	No. of negative results.	Percentage of negative results.
Loeffler's medium .	29	16	55·2	13	44·8
Trypsinised serum-tellurite-agar .	29	19	65·5	10	34·5

All the cultures were virulent for guinea-pigs. It was especially in those swabs from which only a few colonies of *B. diphtheriæ* were obtained on the trypsinised serum tellurite agar that the employment of Loeffler's medium gave a negative result. Before deciding that a culture was negative it was always examined after 24 and 48 hours' incubation.

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## NON-SPECIFIC DESENSITISATION.

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THE phenomenon of non-specific desensitisation in anaphylactic animals has been studied by a number of observers, who have injected widely dissimilar substances to produce it. Thus Biedl and Kraus (1909) employed peptone, Kopaczewski and Vohran (1919) sodium oleate, Friedberger and Hartoch (1909) concentrated saline solutions, while Pfeiffer and Mita (1910) and later Weil (1914) used foreign sera. In a recent paper Karsner and Ecker (1922) have briefly reviewed the literature of this subject and have studied the desensitising effect of various foreign sera on the actively sensitive guinea-pig.

In the course of some experiments on the "anaphylatoxins," Dale and Kellaway (1922) found that normal guinea-pig serum, as well as that treated with starch, agar, etc., when administered intravenously could protect sensitive guinea-pigs from an otherwise fatal dose of antigen injected some time later. It was evident from their experiments that the time which elapsed after the desensitising injection was an important factor in determining the degree of protection conferred. In the experiments to be recorded here we have attempted to analyse this last type of non-specific desensitisation. Incidentally we have produced some further evidence in favour of the cellular view of anaphylaxis.

### THE PROTECTION OF ACTIVELY SENSITIVE GUINEA-PIGS BY INTRAVENOUS INJECTION OF NORMAL GUINEA-PIG SERUM.

Our first concern was to ascertain the extent and duration of the protection afforded to the whole animal. A series of guinea-pigs were injected subcutaneously with 2 mgrm. of crystalline horse serum albumin, which was prepared for us by Dr. H. W. Dudley. Twenty-six days later the lethal dose of antigen was determined for the series as follows:

Weight of guinea-pig.	Dose of antigen (horse serum albumin).	Result.
350 gm.	0.2 mgrm.	Moderate symptoms; recovery in 6 minutes.
310 "	0.5 "	† in 4½ minutes.
260 "	0.5 "	† in 3½ "
280 "	0.5 "	† in 4½ "

The M.L.D. is 0.5 mgrm.

The fresh serum of four normal guinea-pigs was pooled and centrifugated, and 3 c.c. was injected intravenously into each of six remaining anaphylactic guinea-pigs of the above series. At different time intervals after this injection the sensitiveness of these animals was tested by the injection of a dose of antigen. The following results were obtained:

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Weight of guinea-pig.	Time interval after injection of serum.	Dose of antigen (horse serum albumin).	Result.
340 grm.	1 hour	1.0 mgrm.	No symptoms.
295 "	1 "	2.0 "	† in 11½ minutes.
340 "	2 hours	2.0 "	† in 4½ minutes.
340 "	3½ "	1.0 "	No symptoms.
320 "	22 "	0.5 "	Slight symptoms ; recovery.
280 "	22 "	1.0 "	† in 8¾ minutes.

The protection afforded by the injection is not absolute. In this series it is maximal one hour after the injection, when twice the M.L.D. causes no symptoms, and four times the M.L.D. results in a somewhat delayed acute death. Two hours after the injection the protection is somewhat less, and three and a half hours after twice the M.L.D. still fails to produce symptoms. On the day following the injection the protection is greatly diminished, although the animal has not yet completely recovered its original sensitiveness. We now endeavoured to define the part played in the anaphylactic animal by the humeral and cellular defences in causing this temporary loss of sensitiveness. Dale and Kellaway regarded the protection afforded to the sensitive animal by serum containing "anaphylatoxin" and by normal serum as due to a "disturbance of the delicate balance of distribution of antibody between tissue cells and circulating fluid, on the maintenance of which the anaphylactic condition depends." We therefore first attempted to ascertain how the injection of normal guinea-pig serum affected the sensitiveness of the isolated plain muscle.

#### THE SENSITIVENESS OF PLAIN MUSCLE IN ACTIVELY SENSITIVE GUINEA-PIGS AFTER THE INJECTION OF SERUM.

Three series of young virgin female guinea-pigs were actively sensitised by the subcutaneous injection of 1 mgrm. of horse serum albumin. Uniformity of sensitiveness within each series was obtained by avoiding overcrowding and providing a plentiful supply of food during the acquisition of sensitiveness. To test the sensitiveness of the plain muscle the apparatus of Dale and Laidlaw (1912) was used. The two uterine horns, after thorough perfusion by the method of Dale (1913), were separately tested in a bath of oxygenated Ringer of 120 c.c. capacity.

##### *Series 1: Guinea-pigs 38 to 41 days after the Sensitising Injection.*

Of 11 animals of this series, 4 served as controls. As a further control 2 were tested 27 and 30 hours respectively after specific desensitisation by a second injection of 1 mgrm. of the antigen subcutaneously. The remaining 5 were treated with serum as follows: Four large normal guinea-pigs were bled out and the serum was pooled and centrifugated. Of this pooled serum 5 c.c. was injected intravenously into each of the anaphylactic guinea-pigs. Three of these were tested 4, 24 and 27 hours respectively after the injection of the fresh serum, and two were tested 5 and 6 hours after the injection of the same serum when it was 24 hours old. The following results were obtained in the untreated controls:



Weight of guinea-pig.	Dose of antigen (horse serum albumin).	Reaction of plain muscle.
285 grm.	{ 0.02 mgrm.	Small, delayed.
	{ 0.1     "	Maximal.
290     "	{ 0.04 mgrm.	Maximal.
	{ 0.1     "	"
240     "	{ 0.04 mgrm.	<i>Nil.</i>
	{ 0.1     "	Maximal.
290     "	{ 0.04 mgrm.	Large but poorly sustained.
	{ 0.1     "	Maximal.

The sensitiveness of the plain muscle of these control animals was fairly uniform. In all cases a maximal reaction was obtained with a concentration of antigen of 1 in 1,200,000. The individual variation in sensitiveness was shown by the reaction of the second horn, which to a concentration of 1 in 3,000,000 was maximal in one case, modified in another and *nil* in a third. The second horn of the remaining guinea-pig gave a small delayed response to a concentration of 1 in 6,000,000.

In contrast to these normal reactions are those of the three animals treated 4, 5 and 6 hours previously by the intravenous injection of serum.

Weight of guinea-pig.	Time interval.	Dose of antigen.	Reaction of plain muscle.
250 grm.	4 hours	{ 0.1 mgrm.	<i>Nil.</i>
		{ 1.0     "	Barely perceptible.
250 grm.	5 hours	{ 0.1 mgrm.	Moderate.
		{ 1.0     "	Maximal.
200 grm.	6 hours	{ 0.04 mgrm.	Moderate.
		{ 0.1     "	Nearly maximal.

The guinea-pig which was allowed to survive 4 hours was injected with fresh serum, whereas the other two animals received the same serum when it was 24 hours old. The plain muscle of the first showed a marked loss of sensitiveness, reacting hardly at all to a concentration of 1 in 120,000. The uterus of the guinea-pig which was allowed to survive 5 hours after the serum injection showed only a slight loss of sensitiveness. Six hours after the injection of 24-hour-old serum there was no appreciable loss of sensitiveness.

The responses of the plain muscle of guinea-pigs 24 and 27 hours after the injection of fresh serum were as follows:

Weight of guinea-pig.	Time interval.	Dose of antigen.	Reaction of plain muscle.
270 grm.	24 hours	{ 0.04 mgrm.	Delayed, modified.
		{ 0.1     "	Maximal.
230 grm.	27 hours	0.1 mgrm.	Maximal.

The plain muscle at this period after the injection shows no evidence of diminished sensitiveness. In contrast to these results was the specific desensitising effect of the subcutaneous injection of 1 mgrm. of horse serum albumin into animals tested 24 and 27 hours afterwards. Their plain muscle failed to give the slightest trace of reaction to a concentration of 1 in 12,000.

This preliminary series yielded no information concerning the changes in

the sensitiveness of plain muscle occurring earlier than 4 hours after the injection of serum. In the next series we were concerned particularly with these early changes.

*Series 2: Guinea-pigs 63 to 64 days after the Sensitising Injection.*

Of 7 animals in this series 2 served as controls, and the remaining 5 were tested  $\frac{1}{4}$ ,  $\frac{1}{2}$ , 1, 2 and  $3\frac{3}{4}$  hours respectively after the intravenous injection of 5 c.c. of pooled normal guinea-pig serum 24 hours old.

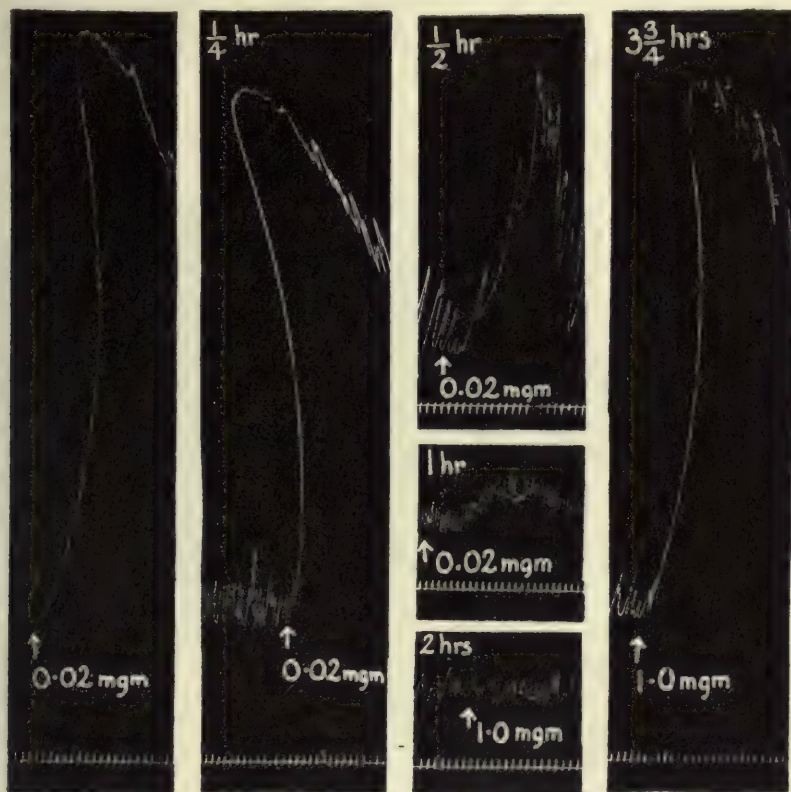


FIG. 1.—Plain muscle reactions of actively sensitive guinea-pigs. Antigen, horse serum albumin. Tracing on left from untreated control; others from animals  $\frac{1}{4}$ ,  $\frac{1}{2}$ , 1, 2 and  $3\frac{3}{4}$  hours after desensitising injection. Time in half minutes.

The control animals gave the following results :

Weight of guinea-pig.	Dose of antigen (horse serum albumin).	Reaction of plain muscle.
330 gm.	0.02 mgrm.	Maximal.
	0.005 "	"
250 "	0.01 "	Maximal.

The plain muscle was highly sensitive, reacting to 1 in 12,000,000 of the antigen. The limits of sensitiveness were not determined.

The following results were obtained after the injection of serum :



Weight of guinea-pig.	Time interval.	Dose of antigen (horse serum albumin.)	Reaction of plain muscle.
290 grm.	$\frac{1}{4}$ hour	{ 0.1 mgrm.	Maximal.
		{ 0.02 "	"
315 "	$\frac{1}{2}$ hour	{ 0.1 mgrm.	Delayed, modified.
		{ 0.02 "	"
290 "	1 hour	{ 0.1 mgrm.	Small
		{ 0.02 "	Trivial, delayed.
350 "	2 hours	{ 0.1 mgrm.	Nil.
		{ 1.0 "	"
300 "	$3\frac{1}{4}$ hours	{ 0.1 mgrm.	Maximal.
		{ 1.0 "	"

The early changes in the sensitiveness of the plain muscle after the injection of serum are well illustrated by this series (see also Fig. 1). In the first  $\frac{1}{4}$  hour after the injection there is no significant change in the reaction of the uterus. Half an hour after the serum injection there is a small change, the plain muscle giving only a modified response to a concentration of antigen of 1 in 1,200,000. One hour after the injection a further small loss of sensitiveness has occurred. Two hours after there is no trace of reaction to a concentration of 1 in 120,000. Three and three-quarter hours after the injection the plain muscle reacts almost maximally to a concentration of 1 in 1,200,000—that is to say, it has regained much of its original sensitiveness.

*Series 3: Guinea-pigs 21 to 24 days after the Sensitising Injection of Horse Serum Albumin.*

Here fresh-pooled guinea-pig's serum was used for desensitisation instead of serum 24 hours old as in the last series. Of seven sensitive guinea-pigs three served as controls, and the plain muscle of the remaining four animals was tested  $\frac{1}{4}$  hour,  $\frac{1}{2}$  hour, 2 hours and 4 hours after the intravenous injection of 3 c.c. of fresh serum.

The plain muscle of two of the controls was tested, with the following results:

Weight of guinea-pig.	Dose of antigen (horse serum albumin.)	Reaction of plain muscle.
210 grm.	{ 0.1 mgrm.	Maximal.
	{ 0.02 "	"
215 "	{ 0.1 mgrm.	Maximal.
	{ 0.02 "	Small, delayed.

The plain muscle was uniformly sensitive to a concentration of antigen of 1 in 1,200,000. Variations appeared in the reaction of the second horn to smaller doses.

The following results were obtained in the guinea-pigs injected with serum (see also Fig. 2):

Weight of guinea-pig.	Time interval.	Dose of antigen (horse serum albumin.)	Reaction of plain muscle.
190 grm.	$\frac{1}{4}$ hour	{ 0.1 mgrm.	Small.
		{ 1.0 "	Slow but maximal.

Weight of guinea-pig.	Time interval.	Dose of antigen (horse serum albumin).	Reaction of plain muscle.
205 grm.	$\frac{1}{2}$ hour	{ 0.1 mgrm.	<i>Nil.</i>
		{ 1.0 "	<i>Small.</i>
205 "	2 hours	{ 1.0 mgrm.	<i>Small.</i>
		{ 10.0 "	<i>Moderate.</i>
205 "	4 "	{ 0.1 mgrm.	<i>Nearly maximal.</i>
		{ 0.02 "	<i>Small.</i>

This series differs from the last in that some change in the sensitiveness of the plain muscle was detected as early as  $\frac{1}{4}$  hour after the injection of serum. The use of fresh instead of 24-hour-old serum may account for this difference. After  $\frac{1}{2}$  hour the plain muscle was very greatly diminished in sensitiveness, giving only a small reaction to a concentration of 1 in 120,000. Two hours after the injection the reaction of the plain muscle was about the same in this concentration, and a moderate response was obtained from the second horn to a concentration of 1 in 12,000. After 4 hours the sensitiveness of the plain muscle had almost returned to its original value.

We may now summarise the changes in sensitiveness

of the plain muscle which follow the intravenous injection of normal guinea-pig serum and compare them with the changes in sensitiveness of the whole animal which we have already described. Little change in the sensitiveness of the plain muscle takes place in the first  $\frac{1}{2}$  hour after the injection, unless fresh serum is used, in which case diminution of sensitiveness appears to occur earlier. After 1 hour desensitisation is well marked, and at the end of 2 hours it appears to have reached its maximum, though it is not complete. Four hours after the injection sensitiveness has returned to somewhere near its original level. Finally, on the following day little or no diminution of sensitiveness can be detected.

The changes in the sensitiveness of the whole animal correspond closely to those of the plain muscle. There is one apparent divergence: 24 hours after the injection the animal still has some protection against the lethal dose of antigen, while at this time the sensitiveness of the plain muscle appears to be completely restored within the limits of individual variation. It is doubtful whether the small change in the sensitiveness of the plain muscle which would correspond to the degree of protection observed in the whole animal at this period could be detected by the methods which we have employed.

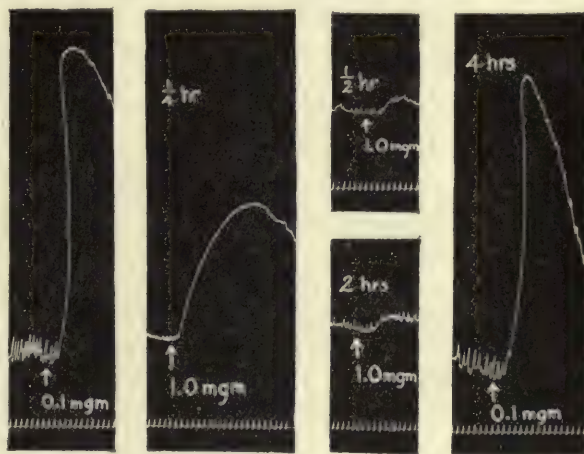


FIG. 2.—Plain muscle reactions of actively sensitive guinea-pigs. Antigen, horse serum albumin. Tracing on left from untreated control; others from animals  $\frac{1}{4}$ ,  $\frac{1}{2}$ , 2 and 4 hours after desensitising injection. Time in half minutes.



There are several possible explanations of the changes in the sensitiveness of the plain muscle :

- (1) The muscle cells may throw off fixed antibody into the circulation.
  - (2) A substance may be formed which inhibits the normal anaphylactic reaction of the animal.
  - (3) The fixed antibody may be destroyed *in situ* and rapidly re-formed.
  - (4) A temporary physical change may occur in the muscle cells which masks the presence of fixed antibody—(a) by preventing the response of the plain muscle to the interaction of antibody and antigen, or (b) by preventing this interaction from occurring.
- (1) Does the injection of serum cause the plain muscle to throw off antibody into the circulating fluid ?

In order to answer this question we investigated a series of guinea-pigs (Series 2) 9 weeks after the sensitising injection. At this period of active sensitiveness, as Weil (1913) <sup>1</sup> and <sup>2</sup> showed, no antibody is demonstrable in the serum, though the plain muscle is highly sensitive. The plain muscle reactions of this series have already been described. The whole of the serum of each of the animals in the series was injected intraperitoneally into a normal virgin guinea-pig, and 48 hours later the sensitiveness of the plain muscle of this guinea-pig was tested. It was found that the serum of the untreated anaphylactic animals failed to confer any passive sensitiveness on the normal animal, the plain muscle of the latter showing no reaction to a concentration of antigen of 1 in 12,000. The serum of anaphylactic animals  $\frac{1}{4}$  hour,  $\frac{1}{2}$  hour, 1 hour, 2 hours and  $3\frac{3}{4}$  hours after the injection of serum likewise failed to produce any trace of passive sensitiveness in their corresponding normal guinea-pigs. These results seem to negative the possibility under consideration, though it may be argued that the method employed is not sensitive enough for the detection of the very small changes in circulating antibody involved. In any case the rapid return of sensitiveness remains to be explained, either by (a) the re-formation of antibody—a possibility which is considered later, or (b) the re-fixation of circulating antibody—a supposition which is rendered improbable by the known facts concerning the time required for fixation of homologous antibody in passive sensitisation.

- (2) Does the injection of serum provoke the formation of a substance which inhibits the normal anaphylactic reaction of the plain muscle ?

If such a substance is formed it must be present within the plain muscle cells themselves, since the blood has been thoroughly washed away from the uterus by perfusion. There is no evidence in our experiments that prolonged perfusion followed by immersion for some hours in repeated changes of oxygenated Ringer causes any alteration in the degree of desensitisation of the plain muscle. If such an inhibitory substance were responsible it is likely that this treatment would cause some restoration of sensitiveness *in vitro*, and in the absence of such evidence it seems unwise to assume the formation of such an inhibitory substance.

- (3) Is the antibody fixed in the plain muscle-cells destroyed and rapidly re-formed ?

The experiments which we have so far described might be explained on this hypothesis. It seemed a little dubious whether, if destruction of antibody

took place in this way, re-formation could occur so rapidly that, with maximal desensitisation at the end of the second hour after the injection of serum, the sensitiveness of the plain muscle could be almost completely restored in the course of the next two hours by the re-formation of antibody. We were able to put this matter to the test of experiment.

Though it is conceivable that such rapid re-formation of *homologous* antibody might occur, any re-formation in the case of *heterologous* antibody is manifestly impossible. We therefore investigated the phenomenon of non-specific desensitisation in guinea-pigs passively sensitised with specific antibody produced in animals of another species.

A rabbit weighing 2460 grm. was immunised by a series of seven injections of from 2 c.c. to 5 c.c. of horse serum at intervals of 7 days. Six days after the last injection its serum diluted five times gave a good precipitate with a 1 in 500 dilution of horse serum. It was now bled out and 2 c.c. of the clear centrifugated serum was injected intraperitoneally into each of a series of guinea-pigs. Forty-four to 48 hours later these guinea-pigs were tested as follows:

Weight of guinea-pig.	Dose of horse serum.	Result.
220 grm.	0.1 c.c.	† in 4 minutes.
300 „	0.02 „	† in 6 „
240 „	0.02 „	† in 6 „
240 „	0.005 „	Slight symptoms; recovery.

Four other animals of this series were injected intravenously with 3 c.c. of fresh normal guinea-pig serum and tested at various periods after the injection, with the following results:

Weight of guinea-pig.	Time interval.	Dose of horse serum.	Result.
250 grm.	1 hour	0.1 c.c.	† in 5½ minutes.
240 „	1½ hours	0.02 „	Very slight symptoms.
300 „	2½ „	0.02 „	Severe symptoms; recovery in 8 minutes.
300 „	4 „	0.02 „	† in 4 minutes.

These animals show no very great loss of sensitiveness, since five times the M.L.D. kills quickly 1 hour after the injection of serum. There is some protection against 1 M.L.D. until 2½ hours after the injection. This loss of sensitiveness has disappeared by the fourth hour. The protecting effect is similar to that observed in actively sensitive animals, appearing and disappearing at corresponding periods.

The plain muscle reactions of two guinea-pigs of this series, investigated at the same period after the sensitising injection, one of which had received an intravenous injection of 3 c.c. of fresh normal guinea-pig serum, were as follows:

Weight of guinea-pig.	Treatment.	Dose of horse serum.	Reaction of plain muscle.
240 grm.	P.S. 64 hours	0.02 c.c.	Moderate.
240 „	P.S. 66 hours	0.01 „	„
	serum injected	0.01 c.c.	Small.
	22 hours	0.1 „	Maximal.



There is but little difference in the reactions; much of the loss of sensitiveness due to the serum injection must therefore have been repaired at this time. While it is just possible that such repair may have been achieved by the formation of homologous antibody, it is much more probable that the return of sensitiveness does not take place by the re-formation of antibody at all. If this be so, it is clear that loss of sensitiveness can be due neither to destruction of antibody nor to its being thrown off into the circulation.

(4) Does a temporary physical change take place in the plain muscle as the result of the injection of serum which masks the presence of fixed antibody?

Failing the discharge of antibody from the cell, its destruction, or the formation of some inhibitory substance, we are left with the hypothesis that some physical change in the muscle-cell is responsible for its loss of sensitiveness. We are not concerned here with any gross alterations in the tonicity of the circulating fluids which would disturb the anaphylactic response of the plain muscle, as has been shown by Dale (1913)<sup>2</sup>. Sensitiveness is not restored to the plain muscle of non-specifically desensitised animals by perfusion with properly balanced saline solutions. Moreover we have found that at the period of maximal desensitisation the isolated plain muscle exhibits its normal rhythm, and is capable of responding with a normal contraction to adequate stimuli, such as small doses of histamine.

The nature of this change, which must be reversible and may be concerned with the degree of aggregation of the cell colloids, and its mode of action, whether by preventing contraction in response to the union of antibody and antigen, by preventing this union, or by diminishing its reaction velocity, must be left for further investigation.

#### THE CHANGES IN THE DEMONSTRABLE ANTIBODY IN THE CIRCULATING FLUIDS OF THE BODY WHICH RESULT FROM THE INJECTION OF SERUM.

The methods which we have applied to the investigation of changes in circulating antibody are less sensitive than those available for the study of fixed antibody. The guinea-pig is not a suitable animal in which to study changes in the titre of precipitin in its serum, and most of our evidence is concerned with somewhat rough attempts to gauge the amount of antibody present by its capacity to transfer sensitiveness to normal guinea-pigs.

#### *Series 3: Experiments with Guinea-pigs 21 to 24 days sensitive.*

The changes in the sensitiveness of plain muscle of these animals have already been recorded. The method of investigating changes in the circulating antibody was to inject the whole of the serum from each animal in the series into a normal guinea-pig and to test its sensitiveness 48 hours later by the intravenous injection of the antigen. The serum of three untreated controls in this series, injected intraperitoneally into normal guinea-pigs, caused the appearance of passive sensitiveness in these latter animals:

Weight of guinea-pig.	Amount of serum injected I.P.	Dose of antigen (horse serum albumin).	Result.
230 grm.	3 c.c.	2.0 mgrm.	Moderate symptoms.
210 "	4 "	5.0 "	† in 4½ minutes.
290 "	4 "	5.0 "	† in 7½ "

The serum of the sensitive guinea-pigs into which 3 c.c. of fresh normal guinea-pig serum had been injected intravenously had lost, at least in part, this capacity for conferring passive sensitiveness. Three such guinea-pigs,  $\frac{1}{4}$  hour,  $\frac{1}{2}$  hour and 4 hours respectively after the injection of serum, yielded  $4\frac{1}{2}$  c.c., 5 c.c. and  $3\frac{1}{2}$  c.c. of serum respectively. The animals injected intraperitoneally with these sera presented no trace of symptoms when 10 mgrm. of horse serum albumin was injected intravenously 48 hours later.

Fifteen minutes after the injection of normal guinea-pig serum there is a noteworthy diminution in the capacity of the serum of the anaphylactic animal to confer passive sensitiveness, which persists until 4 hours after the injection. Dilution of the circulating fluid in the sensitive guinea-pig by the injection of serum cannot be held responsible for this phenomenon, because the whole of the serum obtained from each sensitive animal was injected into the corresponding normal guinea-pig. It is possible that the presence of the serum injected to produce desensitisation, and consequently admixed with the serum which is injected intraperitoneally to produce passive sensitiveness, may prevent such transfer of sensitiveness even if effective antibody is present in sufficient titre. That this is not the explanation of the failure to transfer sensitiveness with the serum of such injected animals can be inferred from experiments recorded in the last section of this paper.

*Series 1: Experiments with Guinea-pigs 38-41 days sensitive.*

The serum of the four untreated controls of this series was pooled, and a concentrated globulin solution (A) was prepared from it in the manner described by Dale and Kellaway (1922). A similar solution (B) was obtained from the pooled serum of the three guinea-pigs which were allowed to survive 4, 5 and 6 hours after the injection of serum. The sera of the two guinea-pigs allowed to survive 24 and 27 hours after the injection yielded the globulin solution (C). These solutions were made up with saline to volumes corresponding to the number of animals from which they were obtained (A) to 8 c.c., (B) to 6 c.c., and (C) to 4 c.c.

Solutions (A) and (B) were centrifugated to free them from suspended particles and their precipitin titres were compared. (A) diluted 10 times gave a faint precipitate against 1 in 5000 of the antigen; (B) treated similarly gave no trace of precipitate in any dilution. We cannot lay any stress on these results because the amount of precipitate with (A) was very small.

The globulin solutions were now tested for their capacity to transfer sensitiveness to normal virgin female guinea-pigs. The plain muscle of these animals was tested 48 hours later, with the following results:

Weight of guinea-pig.	Solution injected I.P.	Dose of antigen (horse serum albumin).	Reaction of plain muscle.
200 grm.	2 c.c. (A)	$\begin{cases} 1\cdot0 \text{ mgrm.} \\ 5\cdot0 \text{ " } \end{cases}$	Small, delayed.
200 "	2 " (B)	10\cdot0 mgrm.	Maximal.
250 "	2 " (B)	10\cdot0 "	Nil.
200 "	2 " (C)	10\cdot0 "	"
200 "	2 " (C)	10\cdot0 "	"



The method used here of testing the plain muscle of female guinea-pigs for passive sensitiveness permits of greater accuracy with a limited number of animals than the method of testing for sensitiveness in the whole animal. From the above experiment it would seem that the capacity of the serum of actively sensitised guinea-pigs for transferring sensitiveness is greatly diminished by the injection of serum, and remains so till the day following the injection.

*Series 4: Immune Guinea-pigs.*

The later changes in the circulating antibody were studied in a group of five immune female guinea-pigs. These animals had received 6 subcutaneous injections of 0.5 c.c. of horse serum at intervals of 7 days, and the experiments were carried out 24 days after the last injection. Two of the animals served as controls, and the remaining three were injected intravenously with 3 c.c. of fresh pooled guinea-pig serum. The controls were bled out and the plain muscle was perfused and tested. It reacted maximally to a dose of 0.1 c.c. of horse serum and gave a good reaction to 0.01 c.c. The guinea-pigs which had been injected with serum were bled out, and their plain muscle was tested 20, 66 and 88 hours respectively after the injection. Their perfused plain muscle gave similar reactions to those yielded by the uteri of untreated guinea-pigs of this series. There was therefore no great difference in the degree of immunisation of the individual members of the series.

Four c.c. of serum from each of these immune guinea-pigs was injected intraperitoneally into a normal female guinea-pig, and after the lapse of 48 hours the passive sensitiveness of the plain muscle of this corresponding series of animals was tested. The following results were obtained:

Weight of guinea-pig passively sensitised.	Serum injected intraperitoneally.	Dose of horse serum.	Reaction of plain muscle.
450 grm.	Untreated immune	0.02 c.c.	Large, poorly sustained.
		0.1 "	Nearly maximal.
350 "	"	0.02 c.c.	Nearly maximal.
250 "	Immune, 20 hours after serum injection	0.1 c.c.	Barely perceptible.
		1.0 "	Small.
300 "	Immune, 66 hours after serum injection	0.1 c.c.	Large, poorly sustained.
		1.0 "	Nearly maximal.
400 "	Immune, 88 hours after serum injection	0.1 c.c.	Large, poorly sustained.
		1.0 "	Maximal.

The serum from the immune guinea-pig 20 hours after the injection of serum showed a well-marked loss of the capacity to transfer sensitiveness, but the serum from animals killed 66 to 88 hours after the desensitising injection was nearly as effective in producing passive sensitiveness as that from the untreated immune animals.

*Summary of the changes in demonstrable circulating antibody after the injection of serum.*—A marked decrease in the demonstrable circulating antibody occurs as early as 15 minutes after the injection of normal guinea-pig serum. There is no evidence of its return to normal until some time between

the 24th and 66th hour after the injection; even after 88 hours it has not been completely restored. We have not yet investigated the cause of these changes. Despite the difference in time relations, it is not likely to be essentially different from that which operates in the case of the plain muscle.

THE DIFFERENCE IN TIME RELATIONS BETWEEN THE CHANGES IN  
DEMONSTRABLE CIRCULATING ANTIBODY AND CHANGES  
IN PLAIN MUSCLE SENSITIVENESS.

In the diagram (Fig. 3) we have represented very roughly the difference in the time relations of these two sets of changes. The titre of antibody is plotted against the time intervals following the injection of serum. The interrupted curve represents the changes in circulating antibody and the continuous one those occurring in fixed antibody.

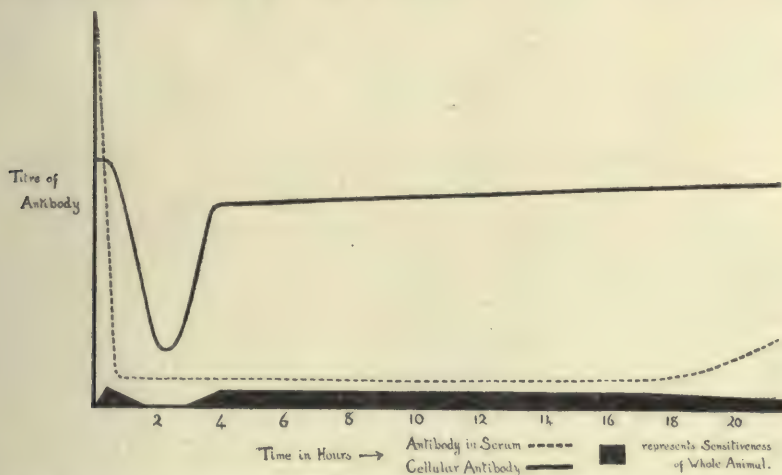


FIG. 3.—Schematic representation of circulating and fixed antibody in an immune guinea-pig. Continuous line = fixed; interrupted line = circulating antibody.

According to Dale and to Weil the anaphylactic reaction is dependent upon the union of antigen with fixed antibody, and the essential difference between immune and sensitive animals is that in the former sufficient circulating antibody is present to unite with the injected antigen and prevent its access to the sensitive cell. The diagram has been drawn so as to represent the effect of the injection of serum in a relatively immune guinea-pig. The titre of circulating antibody is represented as being at a higher level than that of fixed antibody before the injection. If the cellular view of anaphylaxis is the correct one, and if we assume that the antibody in the circulating fluid rapidly loses, as the result of the injection, not only its capacity for the transference of passive sensitiveness, but also, as is suggested by our precipitin experiments, its capacity for union with antigen, it ought to be possible to demonstrate, in any animal with a high titre of circulating antibody, two periods of increased sensitiveness of the whole animal after the injection of serum. Since the changes in the circulating antibody commence earlier than the loss of sensitiveness of the plain muscle, there should be a short period of enhanced



sensitiveness soon after the injection. After the plain muscle has recovered its sensitiveness, *i. e.* after the fourth hour, there should be a second more prolonged period of increased sensitiveness. We have attempted to demonstrate the occurrence of this increased sensitiveness in animals with a high titre of circulating antibody.

*Series 5: Guinea-pigs Actively Sensitised by a Series of Subcutaneous Injections.*

Eight guinea-pigs received a series of 5 subcutaneous injections of 1 mgrm. of horse serum albumin at intervals of 7 days. They were investigated 34 days after the last injection. Similarly treated animals at the same period after the last injection had been found to possess a moderately high titre of circulating antibody. We first determined the M.L.D. of antigen for the series:

Weight of guinea-pig.	Dose of antigen (horse serum albumin).	Result.
350 grm.	0.5 mgrm.	No symptoms.
350 "	0.5 "	"
290 "	1.0 "	† in 3 minutes.

The M.L.D. is 1 mgrm.

The remaining 5 guinea-pigs were injected intravenously with 4 c.c. of fresh pooled serum. Two were tested half an hour after this injection and gave the following results:

Weight of guinea-pig.	Dose of antigen (horse serum albumin).	Result.
290 grm.	0.5 mgrm.	† in 6 minutes.
440 "	0.5 "	† „ 5 „

There was thus a definite increase of sensitiveness following the injection of the serum.

The remaining three animals were tested between the 21st and 24th hour after the injection in order to demonstrate if possible the second period of enhanced sensitiveness:

Weight of guinea-pig.	Dose of antigen (horse serum albumin).	Result.
410 grm.	0.5 mgrm.	Very severe symptoms; only just recovered.
320 "	0.5 "	Severe symptoms; recovery.
500 "	0.5 "	„ „ „

Here a definite increase in sensitiveness was present, though it was not as great as that shown in the early period after injection.

*Series 6: Immune Guinea-pigs.*

This series of animals received six doses of 0.5 c.c. of horse serum subcutaneously at intervals of 7 days. They were tested 24 days after the last injection, when 4 c.c. of serum from one of them injected intraperitoneally into a normal female guinea-pig rendered its plain muscle sensitive to a dose

of 0.02 c.c. of horse-serum. The effect of a dose of 0.5 c.c. of horse-serum on the untreated animals of this series was first tested :

Weight of guinea-pig.	Dose of horse serum.	Result.
390 grm.	0.5 c.c.	Slight symptoms ; recovery.
500 „	0.5 „	„ „ „
450 „	0.5 „	Severe symptoms ; recovery.

The remaining guinea-pigs of the series were injected with 2 c.c. of fresh normal guinea-pig serum. Three of these were tested by the intravenous injection of antigen half an hour after the administration of serum :

Weight of guinea-pig.	Dose of antigen (horse-serum).	Result.
380 grm.	0.25 c.c.	Slight symptoms ; recovery.
360 „	0.5 „	† in 3½ minutes.
440 „	0.5 „	† in 5½ „

The remaining guinea-pigs were tested 20 hours after the serum injection :

Weight of guinea-pig.	Dose of antigen (horse serum).	Result.
410 grm.	0.5 c.c.	† in 30 minutes.
310 „	0.5 „	† in 3½ „
400 „	0.5 „	Severe symptoms.*

There seems to be no doubt concerning the genuineness of this phenomenon of increase of sensitiveness at two different periods after the injection of homologous serum. In this last experiment the increase was not so striking as in the previous one, since 0.5 c.c. of horse serum was probably not much less than the lethal dose of antigen for the untreated guinea-pigs. The small size of the dose of the normal guinea-pig serum in this last experiment may account for this difference in the two series.

In view of these results we must recognise that if circulating antibody is not destroyed after the injection of serum, it loses not only its power of transferring sensitiveness, but also its capacity for union with antigen. In other words, the only two characters possessed by antibody, by which we are enabled to recognise its presence in the circulating fluids of the body, are lost.

The experiments which we have described here are in accordance with the "cellular" theory of anaphylaxis, and are extremely difficult to interpret in terms of the rival theory which attributes the anaphylactic phenomenon to the formation of a hypothetical "anaphylatoxin."

#### CONCLUSIONS.

(1) The intravenous injection of normal guinea-pig serum into actively sensitive guinea-pigs causes a degree of protection against the specific antigen which is only of short duration.

(2) This loss and subsequent return of the sensitiveness of the anaphylactic animal is explained by parallel changes in the sensitiveness of the plain muscle.

\* This animal had apparently recovered at the end of 10 minutes and was then killed. A similar recovery was shown by the guinea-pig which died with circulatory collapse at the end of half an hour.



(3) The changes in the sensitiveness of the plain muscle are probably due to physical changes occurring in the muscle cells.

(4) The demonstrable antibody of the serum is greatly diminished in amount very soon after the injection of serum, and is not restored till long after the sensitiveness of the plain muscle has returned to its original value.

(5) The injection of guinea-pig serum into guinea-pigs with a high titre of circulating antibody is followed by the appearance of enhanced sensitiveness of the whole animal during two periods—one shortly after the injection, and a second after the restoration of the sensitiveness of the plain muscle but before the return to normal of the demonstrable circulating antibody.

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### ON THE OCCURRENCE OF A TOXIC SUBSTANCE IN THE BLOOD IN CASES OF BRONCHIAL ASTHMA, URTICARIA, EPILEPSY, AND MIGRAINE.

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It has become clear during the last few years that illnesses like asthma, hay fever, urticaria, etc., have, in most instances, to be considered as "allergic diseases," which means that these pathological conditions are due to hypersensitiveness to substances which are innocuous for normal people. These substances, causing attacks of asthma or hay fever or urticaria, may be pollen, or some animal protein, or some foodstuff or drug, or, as highly interesting experiments of Cooke (1922) have shown, an unknown substance occurring in "house" dust.

Without any doubt this conception holds good for most cases of asthma and allied diseases, and consequently it can be stated that in the greater

number of these cases the direct cause of the attack is known. Since the agents causing an attack in certain individuals are completely innocuous for normal persons, it must be surmised that asthmatics, sufferers from hay fever, urticaria and so forth possess a certain "disposition" to allergic reactions. Of the nature of this "disposition" or "allergic condition" little is known. An attempt has often been made to demonstrate that this condition is an anaphylactic one, but this view, as has been emphasised by Coca, Cooke and others, must be abandoned. The allergic condition may be related to anaphylaxis, but it is not a true anaphylactic phenomena, such as occurs in animal experiments.

Moreover, it is known that in allergic diseases hereditary factors play an important rôle. Cooke and van der Veer (1916) find among 504 cases of hay fever hereditary antecedents in 48·4 per cent.

de Kleyn (1912), in collaboration with one of the present writers, showed that most asthmatics present a disordered purine excretion, whilst Storm van Leeuwen and Varekamp (1921) drew attention to the fact that a great number of sufferers from asthma and hay fever give a considerable v. Pirquet tuberculin reaction, and are highly benefited by tuberculin treatment. In addition it is known that in allergic conditions there mostly is a hypersensitiveness to *several* agents; more than one animal protein, animal protein + pollen, animal protein + aspirin, and so forth. Such patients are apt to be of a nervous disposition, so that there is considered to exist a hyperexcitability of the sympathetic system.

All these things are of considerable interest, but they do not offer a satisfactory explanation of the mechanism of allergic disposition.

In view of this state of affairs the present authors felt justified in facing the problem from a different angle, and they adopted the following working hypothesis.

Apart from all other considerations stated above, the asthmatic condition is—or, generally speaking, allergic conditions are—due to a hyperexcitability of smooth musculature in the lung (or other organ). *This hyperexcitability is due to a substance present in the blood of these patients, a substance which has the property of stimulating smooth muscle.*

The testing of this hypothesis seemed simple at first. Blood of a number of asthmatics was taken, and allowed to clot. After 24 hours the serum was taken off and its action on isolated organs was tested. It has long been known that normal serum contains substances which stimulate smooth musculature; and recently one of the present authors and his co-workers (Storm van Leeuwen, 1921) showed that it also contains substances which are able to augment the action of certain drugs. This action of normal serum of course offered a difficulty in the testing of sera of pathological cases, but we hoped to be able to show that the serum of an asthmatic would be more effective in one or the other way than normal serum. However, we were disappointed in this respect, for although the serum of asthmatics was, as a rule, highly active, it was not more active than some normal human sera. It therefore became necessary to devise a method which would enable the stimulating and augmentor substances occurring in normal serum to be removed. Our attention was then drawn to a recent publication of Freund



(1921), who, with a different purpose, was faced with the same problem, viz. the elimination of the active substances of normal serum. It is conceived that these substances are not present in the uncoagulated blood, but only form during clotting. Experiments of Trendelenburg (1916), however, have shown that although freshly drawn blood is free from these substances, they appear, even if clotting is prevented by sodium oxalate, within half a minute after the blood has been shed. Starting from this fact, Freund worked out the following method: Blood is drawn from a large vein with a thick needle, and allowed to flow freely into a flask containing 96 per cent. alcohol. The alcohol is then evaporated off at low temperature, and the residue taken up in physiological saline. This solution if obtained from normal individuals does not contain stimulating substances.

It seemed to us that Freund's method might prove useful for our purposes and we performed the following experiments:

Blood was drawn, in the way described by Freund, from 23 men, viz. 10 normals, 9 asthmatics, 2 sufferers from intense urticaria, 1 epileptic and 1 case of migraine.

From every sample of blood an alcoholic extract was made and all the extracts were tested on the isolated gut of the cat. We chose this procedure because it has proved in our hands to be one of the best and most convenient methods for studying the action of drugs on smooth musculature. In all experiments the sensitiveness of the gut for pilocarpine was first tested, and only after a constant sensitiveness to this drug was established was the blood extract to be tested added. This enabled us (1) to calculate roughly the amount of stimulating substance present in a certain blood, comparing its effect with that of pilocarpine, and (2) to judge whether in a given extract substances might be present which show an augmentor effect on pilocarpine action.

Briefly stated the results of these experiments were as follow:

The extracts of all the normals showed no action at all on the gut, if doses corresponding to 4-12 c.c. of blood were used.

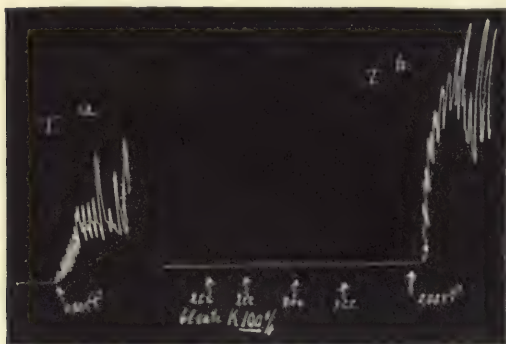


Fig. 1.—Extract of normal blood has no influence on isolated gut. a. 0.005 mgrm. pilocarpine hydrochloride added to 75 c.c. tyrode solution gives moderate contraction of gut. b. 10 c.c. blood extract corresponding to 10 c.c. blood from normal man gives no contraction; 0.005 mgrm. pilocarpine subsequently given produces strong contraction.

The extract of the blood of one asthmatic (very light case who had only attacks occasionally) was also negative. *In all the other cases of asthma and in the cases of urticaria and migraine the blood extract contained a substance which had a very definite action on the smooth muscle of the gut.* Roughly calculated 1 litre of blood contained a quantity of the unknown poison which had an action similar to that of 2-5 mgrm. of pilocarpine. The slight cases had less of this poison in the blood than the severe cases. In the one case of epilepsy which was studied only a small amount of the toxic substance was found. Figs. 1 to 3 give instances of the experiments described, whereas

Tables I and II give the results of all the experiments made.

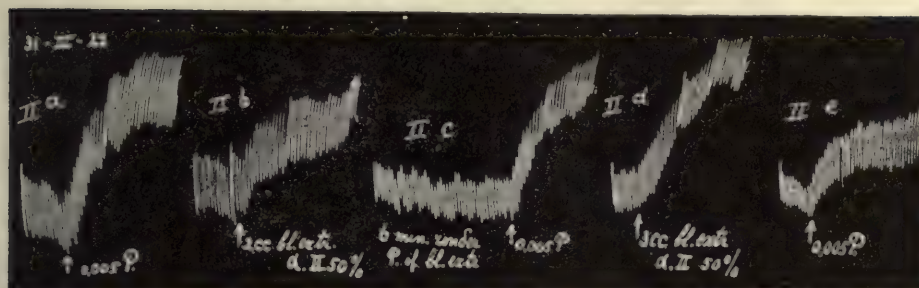


FIG. 2.—Influence of blood extract from severe case of asthma on isolated gut. *a.* 0.005 mgrm. pilocarpine gives moderate contraction. *b.* 2 c.c. blood extract corresponding to 1 c.c. blood gives contraction of gut. *c.* Action of 0.005 mgrm. pilocarpine. *d.* Action of 3 c.c. blood extract corresponding to  $1\frac{1}{2}$  c.c. blood. *e.* Action of 0.005 mgrm. pilocarpine.

TABLE I.

Exp.	Diagnosis.	Blood extr. in c.c.		Sensitiveness of the gut for pilocarpine in mgrm.
A	Nephritis	5	No stimulation	0.005 mgrm. ++
B	Nil	4	"	0.005 " +
C	Trachei-bronchitis	4	"	0.002 " ++
D	Lues (W. R. ++)	4	"	0.002 " ++
E	Nil	4.5	"	0.002 " ++
F	"	5	"	0.005 " +
G	Lues (W. R. +)	5	"	0.005 " +
I	Nephritis	7	"	0.005 " ++
K	Tuberculosis pulm.	10	"	0.005 " ++
M	Tumour mediastini	12	"	0.005 " ++

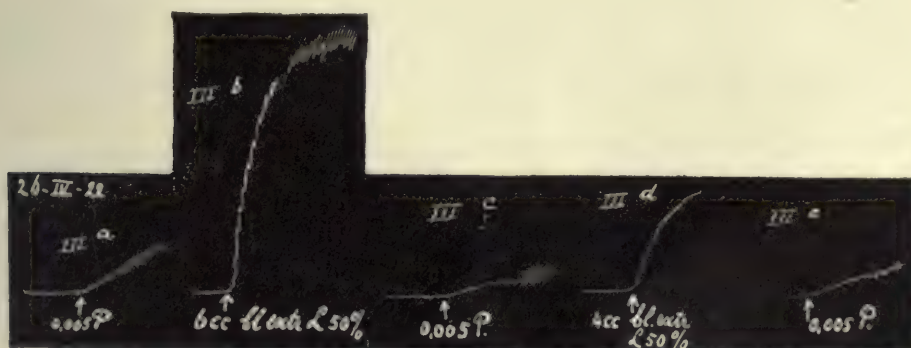


FIG. 3.—Action of blood extract from a severe case of urticaria. *a.* Action of 0.005 mgrm. pilocarpine. *b.* Action of 6 c.c. blood extract corresponding to 3 c.c. blood. *c.* Action of 0.005 mgrm. pilocarpine. *d.* Action of 4 c.c. blood extract corresponding to 2 c.c. blood. *e.* Action of 0.005 mgrm. pilocarpine.



TABLE II.

Exp.	Diagnosis.	Stimulation substance in 1 litre blood corresponding to mgrm. of pilocarpine.
aI	Bronch. asthma	No stimulation.
aII	"	$\pm$ 5 mgrm.
aIII	"	$\pm$ 3.33 mgrm.
aIV	"	$\pm$ 3.33 "
aV	"	$\pm$ 2.5 "
aVI	"	$>$ 2.5 "
aVI	"	$<$ 5 "
aVII	"	$\pm$ 5 "
aVIII	"	$\pm$ 5 "
aIX	"	$\pm$ 2 "
H	Epilepsy	$<$ 1.66 "
L	Urticaria	$>$ 2.5 "
N	Migraine	$>$ 2.5 "
Q	Urticaria	$\pm$ 4 "

The finding of a toxic substance in human blood is not new. Many years ago it was shown that the blood of cases of epilepsy or eclampsia (Graf and Landsteiner, 1909) is more toxic for animals than normal blood. Recently Freund and Gottlieb (1921) found a poison in the blood of diseased animals and in certain human illnesses. The importance of the findings related in the present paper lies in the fact that a poison can be demonstrated in the blood of certain individuals during periods of apparently good health. So this toxic substance does not occur during an acute illness, but it seems to be an indication of a "pathological state," or of an "allergic disposition."

## DISCUSSION.

The writers are quite well aware that the fact presented here, viz. the occurrence in blood extracts of twelve sufferers from allergic diseases of a toxic substance not present in normal blood extracts, although of considerable interest, by no means solves the problem of the mechanism of allergic conditions or asthmatic disposition. On the other hand it opens a new field for investigation where the clue to the problem might be found. Further work on the subject is in progress in this institute.

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## APPLICATION OF THE ABSORPTION OF AGGLUTININ TEST TO THE SEROLOGICAL STUDY OF PNEUMOCOCCI.

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THE pioneers of the serological classification of disease-producing pneumococci in New York (Avery, 1917) found that a number of strains titrated against Type II anti-serum exhibited agglutination, but only in lower dilutions and after more prolonged exposure than typical strains.

An analysis by myself of 53 cultures, derived not only from lobar pneumonia, but from a variety of other acute inflammatory conditions due to the pneumococcus, showed that the Type II group was very prevalent in London in the winter of 1919-20. The series included 16 strains obtained from cases of lobar pneumonia in adults, of which half were due to Type II infection and the remainder to Type I—a relative incidence of type corresponding to accepted statistics. Type II infection was responsible for the majority—73 per cent.—of the strains from all sources examined, a much higher relative incidence, but it was not found possible accurately to discriminate in every case by agglutinating titre and time response between typical and atypical strains of Type II, and the necessity for applying the further test of absorption of agglutinin in subsequent experiments became very apparent.

Considerable difficulty was experienced in 1919-20 in the production of a Type II anti-serum of good agglutinating power and scope—a difficulty also at first encountered in America. This was overcome after repeated trial of different strains by the discovery of a typical strain of good antigen content, isolated by blood culture *post mortem* from a fatal case of lobar pneumonia in an adult man.

The technique of the absorption test used in the experiments which form the subject of the present paper was as follows: 1 c.c. of an emulsion of the organism to be tested, of strength 8000 million cocci per c.c. (estimated by the opacity of the emulsion compared with a standard) was mixed with an equal bulk of serum diluted 1 in 5, and incubated for 2 hours in a water-bath at 37° C. in centrifuge tubes, the contents being shaken after 1 hour to secure complete admixture. The serum employed had agglutinating power up to dilutions of 1 in 80, and the above strength of emulsion of coccus was found by experiment to be sufficient to absorb completely the agglutinin present. The contents of the tubes were centrifuged after incubation, 15 minutes usually sufficing to precipitate the suspension completely, and the clear serum removed by pipette and titrated against an emulsion of the homologous standard type coccus. The absence of any agglutination after incubation for 24 hours at 55° C. indicated total absorption of agglutinin and proved the strain to be a true homologue of the type coccus.



TABLE I.—*Serological Relationship of Type to Sub-type Pneumococcus Strains.*

A. Investigation of the effect of saturating a Sub-type I serum with corresponding Sub-type I and Type I coccus on the agglutinating power of the serum for its homologous coccus, with controls.

*Conditions.*—Saturation for 2 hours at 37° C. in the water-bath; centrifugalisation 15 minutes; clear, supernatant serum pipetted off and titrated against homologous coccus. Final titres read off after 12 hours' incubation at 55° C.

Stage I.—Saturation.	Stage II.—Titration of serum after saturation against an emulsion of the homologous sub-type coccus.				Result.
	Serum dilutions.				
	20.	40.	80.	160.	
Sub-type coccus suspended in normal saline solution	—	—	—	—	No agglutination. Emulsion satisfactory.
Sub-type coccus emulsion in presence of normal serum	—	—	—	—	No agglutination by rabbit's normal serum.
Control agglutination of standard Type coccus by the sub-type serum	—	—	—	—	Absence of agglutination.
Control agglutination of sub-type coccus by homologous unsaturated serum	++	++	+	—	Serum titre determined.
Control agglutination after saturation with an indifferent coccus—Type II	++	++	+	—	Agglutinating power of serum unaltered.
Sub-type serum after saturation with homologous coccus	+	—	—	—	Almost complete absorption of agglutinin.
Sub-type serum saturated with standard Type coccus	++	++	+	—	Agglutinating power of serum unaltered.

B. Absorption of Sub-type agglutinin from standard Type serum by Sub-type and standard coccus. Titration against Sub-type coccus as in A.

Test agglutination of sub-type coccus by Type I serum	++	++	++	++	Serum titre determined.
Serum after saturation with sub-type coccus	—	—	—	—	Complete absorption of sub-type agglutinin.
Serum after saturation with standard Type I coccus	—	—	—	—	Complete absorption of sub-type agglutinin.

C. Effect on standard agglutinin of preliminary saturation with Sub-type coccus. Titration with standard coccus after saturation.

Control agglutination of Type I coccus by Type I serum	++	++	—	—	Serum titre determined.
After saturation with sub-type coccus	++	++	—	—	Agglutinating power of serum unaltered.

+ + = Complete agglutination, clear fluid. + = Distinct agglutination, fluid not clear.  
— = No agglutination.

The importance of controlling the experiment at each stage cannot be insisted upon too strongly, and is fully indicated in Table I. The behaviour of the homologous coccus and of a heterologous strain in the presence of the homologous serum before saturation of the latter, and likewise the behaviour of the unknown strains under investigation in the presence of rabbit's normal serum, have alike to be re-tested in each experiment, under conditions precisely similar to those to which the unknown strains are subjected. By this means the possibility of error due to variation in the behaviour of serums from time to time or spontaneous agglutination of an emulsion is excluded. The serums used have been prepared by rapid inoculation of rabbits, using the method described in a previous communication, viz. intravenous inoculation at intervals of 48 hours, with ascending doses of pneumococcus emulsion in phenolated saline solution; doses range from 2000 million to 10,000 million and upwards. Five or at most six inoculations usually suffice to produce a serum capable of completely agglutinating emulsions of the homologous coccus (strength 4000 million cocci per c.c.) in dilutions of 1 in 80, after 24 hours' incubation at 55° C. in the hot-air incubator. Such serums also agglutinate fully in dilution 1 in 40, and rather less completely to 1 in 80, within two hours in the water-bath at 37° C. Emulsions of coccus are obtained by suspending in 0.85 per cent. saline the surface growth on human blood legumin agar plates, after 12-16 hours' incubation, the suspensions being heated to 60° C. in the water-bath for 20 minutes to destroy autolysin. The emulsions are kept free from bacterial growth by addition of phenol to 0.5 per cent.

In the course of the present inquiry strains of pneumococci have been collected from many sources, embracing both hospital and general practice, and these may be considered fairly representative of the incidence of the several types. In establishing the identity of particular strains with true pneumococci, the accepted criteria of capsule formation, bile solubility and cultural characters have been rigidly observed, and results of plate cultures in many cases have been confirmed by mouse passage.

The absorption test has been applied to cocci of all three groups, with the result that the relationship of the standard Type II pneumococcus to its sub-types has been defined, and in addition the hitherto unsuspected existence of aberrant strains related to Types I and III has been discovered. The numerical frequency of each and their occurrence in disease is set out in Table III, based on an analysis of 200 strains obtained during 1920-22.

Sub-types of all three main types have been found responsible for lobar pneumonia.

The avoidance of confusion in the serological classification of pneumococci presents some difficulty. The position may be defined as follows: There are three or, with the possible addition of Lister's A strain, four pneumococcus types responsible for the majority of the most characteristic forms of pneumococcus infections in man—lobar pneumonia, empyema thoracis, meningitis and peritonitis. In addition an unknown number of strains exist, hereinafter described as sub-types, corresponding to each of the chief types. These sub-types possess in common the property of agglutinating with their corresponding "type" serum but not with serums homologous with other "types." A sub-type, although more closely related to its corresponding "type" than to other pneumococcal strains, is in other respects distinct from it. In the case of the sub-types of Type II, several strains serologically identical have been found in the case of each sub-type, but it is not yet possible to define the number of separate sub-types either of Type II or of Types I and III which exists. The serological characters of sub-type strains are described in greater detail below.

It is much to be regretted that the Rockefeller staff permitted themselves



to apply the loose term "Type IV" to the unclassified and ill-defined pneumococcus strains, which, although isolated from definite morbid sources, failed to agglutinate with any of their three representative serums. By so doing they have by implication closed the door for the admission of any other type of pneumococcus to the series. There seems strong reason for believing that Lister's Type A represents such an addition, and the question naturally arises how this organism may be recognised in future—if as Type V what strain shall be accepted as Type IV pneumococcus?

Table I illustrates the serological relationship of Type I pneumococcus and its allies. Type I is chosen as an example; the relationship of Types II and III to their sub-types appears to be similar.

The table shows that a sub-type coccus is agglutinated by and completely absorbs its own agglutinin, both from its own serum and the type-serum. Saturation of type-serum with sub-type coccus, however, in no way affects the agglutinating power of type-serum for type-coccus, or for other dissimilar sub-type strains. Apparently, therefore, the sub-type antigens can be removed piecemeal from the standard serum without in any way affecting its agglutinating power for the standard coccus or for other, dissimilar, sub-strains. Type I coccus is not agglutinated by a serum prepared against a "sub-type" coccus and has no power of absorption of sub-type agglutinin, but absorbs all the sub-type agglutinin as well as its homologous agglutinins from the standard serum, depriving this of all agglutinating power against either type or sub-type. These results are identical with those obtained by Castellani (1902), working with the typhoid-colon group, who proved that the agglutinins of any related strain can be removed from the standard serum by fractional absorption with their homologous strain, leaving the anti-bodies for similar strains and for the standard strain intact. No relationship between any of the sub-type strains of Type I has yet been discovered, each sub-type so far having exhibited specific antigenic qualities.

Of the seven strains agglutinated by Type III serum, investigated in the present inquiry, two were found incapable of absorbing the standard agglutinin. An anti-serum prepared against one of these was proved specific for its homologous coccus, and behaved in a manner strikingly comparable with the serums from sub-type I strains described above.

The technique employed has sharply delimited the standard Type II pneumococcus from the numerous strains which agglutinate with but do not absorb Type II serum. In view of the apparent complexity of this group the following statements based on the experiments may be made:

(1) The standard Type II pneumococcus contains an antigen, which is capable of eliciting in rabbits the formation of agglutinins, containing not only the factor capable of agglutinating the homologous standard coccus, but also rich in sub-agglutinins which react with atypical cocci. These atypical cocci are sharply differentiated from typical members of the group by failure to absorb the standard agglutinin.

(2) The higher the titre of the standard type anti-serum the richer and less specific appears to be its sub-agglutinin content. Titration experiments demonstrate that the titre for sub-strains is seldom so high as that for the truly homologous type-coccus.

TABLE II.—Confirmation of Types, provisionally determined by Agglutination, by the Absorption Test.

Reference.	Source.	Type-serum dilutions.														Normal Serum.	Inference.	Absorption of Type agglutinin.
		Type I.				Type II.				Type III.								
		10.	20.	40.	80.	10.	20.	40.	80.	10.	20.	40.	80.					
Type I	Meningitis	++	++	—	—	—	—	—	—	—	—	—	—	—	—	—	Homologous	Complete.
Type II	Lobar pneumonia	—	—	—	—	++	++	++	+	—	—	—	—	—	—	—	No cross	Complete.
Type III	Lobar pneumonia	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Agglutination	Complete.
49	Lobar pneumonia	++	+	—	—	—	—	—	—	—	—	—	—	—	—	—	Type I	Complete.
15	Lobar pneumonia	++	++	++	++	—	—	—	—	—	—	—	—	—	—	—	Type I	No absorption = sub-type
52	Lobar pneumonia	—	—	—	—	—	—	—	—	++	—	—	—	—	—	—	Type II	Complete.
50	Chronic bronchitis	—	—	—	—	—	—	—	—	++	—	—	—	—	—	—	Type II	No absorption = sub-type.
48	Lobar pneumonia	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Type III	Complete.
2	Acute mastoid	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Type III	No absorption = sub-type.
7	Lobar pneumonia	++	++	++	+	++	++	++	++	++	—	—	—	—	—	—	Uncertain	Complete of Type II.



(3) Application of the absorption test to pneumococci belonging to the sub-types of Type II demonstrates a relationship between type and sub-type similar to that which exists between Types I and III and their subordinates. No anti-serum prepared against a sub-type II pneumococcus has possessed any agglutinating power for the standard Type II organism.

(4) The majority of strains of sub-type II obtained in the course of the inquiry were unrelated to one another, for no cross-absorption of agglutinin from sub-type serums occurred, but, in a few examples, identity, as proved by the absorption test, was found to exist. The similarity of these sub-types to Stillman's 2A and 2B has not been proved, nor have cultures of these strains, obtained from the National Collection of Type Cultures, exhibited agglutination either with any of the sub-type II serums prepared by myself, with my standard Type II serum, or with a Rockefeller Type II serum obtained direct from New York, but Griffith (1921) has recovered examples of both 2A and 2B from cases of lobar pneumonia in this country. It is possible that prolonged sub-cultivation *in vitro* has impaired the properties of the strains.

(5) An anti-serum was prepared by immunisation of rabbits against each of several of the sub-type II strains isolated in the course of the inquiry. Each serum was found to be possessed of agglutinating power, not only for other sub-strains, but also for strains previously exhibiting no reaction with any of the three standard sera. In addition these sub-type serums were found to agglutinate strains of organisms isolated from such trustworthy sources as lobar pneumonia sputums, before or immediately after crisis, which strains, although bile-soluble, differed in some of their characters from true pneumococci. These strains appear to be intermediate between pneumococci and the salivary streptococci.

(6) Furthermore, strains undoubtedly belonging to the group of salivary streptococci and culturally approximating to the descriptions of Schottmüller's *Streptococcus viridans*, show, when recently isolated from inflammatory exudates, feeble agglutination with Type II serums; but these strains differ markedly from the non-agglutinating and transitional forms of pneumococcus described above, in that they have vigorous agglutinogenic power when inoculated into rabbits. Serums with a titre of 1 in 640 can be prepared with ease by half-a-dozen graduated doses of such cocci at 48-hour intervals, affording the sharpest contrast with the pneumococcus group, which is notoriously of low agglutinogenic power. Two of these strains were selected for immunisation of rabbits, and the serums tested on a small group of twelve similar strains. One serum agglutinated its homologous coccus only, the other agglutinated two other strains as well as its homologue.

(7) So far as investigated, sub-type II pneumococci have lower agglutinogenic power than the standard organism. In serums prepared from them there is more rapid deterioration of agglutinin on keeping than in the case of standard serum, which maintains its agglutinating power remarkably well under all conditions of exposure, specimens under observation for two years having depreciated only 50 per cent. when stored in a cold and dark place. In addition, certain sub-type strains have been found, after repeated sub-cultivation, to lose the property of agglutinating with standard serum. As stated above, this observation seems to apply also to the cultures of Rockefeller Types

2A and 2B obtained from the National Collection of Type Cultures, mentioned in paragraph (4).

(8) From time to time well-authenticated strains of pneumococcus exhibit the phenomenon of agglutination in presence of both Type I and Type II serums but not in the presence of normal rabbits' serum; the absorption test has proved these invariably to be Type II pneumococci.

(9) These observations indicate that the agglutinins produced in the serum of rabbits in response to inoculation with Type II pneumococcus have much wider scope than those which result in the case of Types I and III.

TABLE III.—*Serological Analysis of Disease-producing Pneumococci, 1920-22.*

Disease.	No. of strains of the different types.								
	I.	I. Sub- types.	II.	II. Sub- types.	III.	III. Sub- types.	Un- classified.	Non- specific.	Total.
Lobar pneumonia . . .	20	4	21	5	3	2	9	6	70
Broncho-pneumonia, children . . .	7	—	—	10	—	—	12	2	31
Bronchitis, adults . .	8	—	—	4	—	—	8	3	23
Influenzal pneu- monia, associated <i>B. Pfeiffer</i> . . .	1	—	—	—	—	—	2	4	7
Empyema . . .	14	—	—	—	—	—	—	4	18
Fauces, sore throat, pure culture . . .	2	1	—	—	—	—	3	—	6
Nasal catarrh, pure culture. . . . .	7	—	—	4	1	—	12	4	28
Meningitis . . .	4	—	—	—	—	—	—	4	8
Pericarditis (child) .	—	—	—	—	—	—	1	—	1
Middle-ear. Otitis .	1	—	—	—	1	—	1	1	4
Conjunctivitis . . .	1	—	—	—	—	—	—	—	1
Peritonitis . . .	3	—	—	—	—	—	—	—	3

Strains described as "unclassified" fail to agglutinate with any of the serums employed.

"Non-specific" strains agglutinate indiscriminately with any pneumococcus anti-serum, but exhibit no absorption of specific agglutinin from any.

(10) It follows from the above observations that agglutination readings unsupported by a qualifying absorption test are an insufficient guide to type, and in not a few instances may be actually misleading as a therapeutic index. The protocol of an experiment in which agglutination results were corrected by the absorption test exemplifies this point (Table II).

Although carried out entirely independently, the above results are substantially identical with those obtained by Avery (1915) in his investigation of 10 strains of aberrant Type II pneumococci. Unfortunately, my experiments lack the complete proof which virulence tests would have afforded and



which Avery was able to supply. His study of the protective power of anti-serums, prepared against members of the sub-types of Type II, confirms and extends the results arrived at by means of agglutination and absorption tests. Of particular importance is his observation that saturation of the standard serum by its homologous coccus removes all the agglutinins and protective bodies, while absorption by a related sub-type strain does not materially affect these.

It may not be altogether out of place to review here the significance of the added complexity of type revealed by the experiments summarised above. Avery clearly demonstrated by immunological methods that the standard Type II serum has protective power against organisms of the two sub-groups 2A and 2B, although not against the mixed coterie of strains described by him as Group 2X. The serological affinities between the typical and atypical members of Types I and III, identified and described by me, appear to be of the same order as those which exist between Type II and its sub-types A and B.

If this be confirmed by subsequent experiment, it may well be that infection by sub-types I and III is, in a similar manner, amenable to treatment with corresponding standard serums.

The phenomenon of macroscopic agglutination is a two-phase reaction :

- (a) Conjugation of coccus and agglutinin.
- (b) Precipitation in presence of an electrolyte.

Theoretically it is possible that the agglutination reaction may at times be incomplete, in the sense that stage (b) does not ensue. To test this hypothesis type-serums have been saturated with non-agglutinating (unclassified) strains of pneumococcus in order to ascertain if absorption of agglutinins occurs such as might indicate an occult relationship to type. In every case, using each of the three standard serums, the results have been negative, *i.e.* phases (a) and (b) were both absent.

The numerous non-agglutinating strains of pneumococcus variously described as Group IV and sometimes improperly as Type IV are, in my experience, conspicuously deficient in antigen for rabbits; in one example in which a series of doses of killed vaccine were followed by inoculation with living culture, resulting in a septicæmia of ten days' duration, only the feeblest agglutinating power developed, even when undiluted serum was employed.

The experiments described have been carried out in the Research Laboratory of St. Bartholomew's Hospital by permission of Sir Frederick Andrewes, to whom, and to Dr. H. M. Gordon, Bacteriologist to the Hospital, I am greatly indebted for much practical advice and help.

The work was undertaken with the assistance of the Medical Research Council, to whom my grateful thanks are due.

#### CONCLUSIONS.

(1) Agglutination by specific pneumococcal serums is not invariably sufficient for recognition of type. The absorption of agglutinin test affords satisfactory confirmation.

(2) The use of the absorption test has led to the identification of sub-types of Type I and Type III pneumococcus comparable to the sub-types 2A and 2B of Type II already differentiated by Avery.

(3) Sub-type strains exhibit limited specificity within the group to which they belong; in no case has a serum prepared against a sub-type pneumococcus agglutinated the standard type strain.

(4) The serum of rabbits inoculated with the standard Type II pneumococcus is particularly rich in subordinate agglutinins. This property is shared by sub-type 2 strains in general, since the sub-type serums were found to agglutinate pneumococcus strains which had failed to react with any of the three standard serums. By this means a degree of serological relationship between the Type II group and salivary streptococci of the *S. viridans* group has been demonstrated. Salivary streptococci are sharply differentiated from pneumococci by their vigorous agglutinogenic properties.

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## THE INFLUENCE OF THE QUALITY OF THE MEAT USED UPON THE REACTION CURVE OF A NUTRIENT MEDIUM.\*

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PREVIOUS investigations into the type of meat most suitable for bacterial metabolism have referred chiefly to the development of the maximum toxigenic power of *B. diphtheriae* in nutrient broth. The presence of fermentable substances was by some thought undesirable owing to the production of acid. Th. Smith (1895), however, while endeavouring to avoid "muscle sugar," thought that small quantities of glucose were beneficial.

In the present paper the quantity of glucose in meat is determined and the effect of different meat samples upon the reaction curve of the medium during growth of *B. diphtheriae*.

#### THE AMOUNT OF GLUCOSE IN MEAT INFUSIONS.

Cadeac and Maignon (1902) published sets of analyses of various muscle tissues for carbohydrates, but an examination of their results does not allow one to draw any very certain conclusions. They used Fehling's solution to

\* A Report to the Medical Research Council,



estimate the sugar in the bouillon "après défécation." It is difficult to eliminate the extent to which nitrogenous substances were responsible for reduction. They found the greatest concentration of glucose in heart muscle. Smith (1895) estimated that the glucose in meat varied from a trace to 0.3 per cent. Fresh meat contained less than that which has been allowed to stand. This statement is remarkable in view of the modern idea of glycolysis, yet the present experiments appear to bear out what Smith asserted.

There is little other information of value regarding the concentration of glucose in muscle tissue.

In a survey of the methods which would be available for the estimation of amounts of glucose of the order supposed to be in bouillon that of Benedict was chosen. Previous experience with the method of Dufau and Patein of precipitation with mercuric nitrate had shown that this removed quantitatively such substances as creatinine, which might in the subsequent digestion give a colour with alkaline picrate solutions. I have further also found that glucose was quantitatively recovered from urines to which it was added after precipitation with this reagent.

For the purpose of the present experiments, weighed quantities of meats freed from fats as completely as possible and finely minced were placed in somewhat more than their weight of cold water. The mixture was raised slowly to 80°C. It was then filtered through paper and allowed to drain well and the amount of filtrate measured. An aliquot portion of the filtrate was used for analysis, the volume being referred to the amount of meat originally used. The procedure was essentially that described by Benedict. The picric acid used had been recrystallised three times by Folin's method. The meats used were: (1) English beef, freshly killed; (2) imported beef; (3) bullock's heart, English freshly killed.

The following results were obtained:

English beef . . .	0.039 per cent. glucose.
Foreign beef . . .	0.073 " " "
Heart . . .	0.0 " " "

In order to see if the colours produced by the alkaline picrate were due to fermentable substances, the meat infusion in each case was heated with fresh bakers' yeast at 37°C. for 12 hours, and the mixture treated with mercuric nitrate and picric acid as before. No colour developed in any of the samples. The colour was therefore due in the first instance to a fermentable substance.

The results with heart muscle were so contrary to expectations that the whole experiment was repeated, using pig's heart. In addition a portion of bullock's heart was obtained from the slaughter-house and worked up in less than an hour from the time the animal was killed.

The following results were obtained:

English beef, freshly killed . . .	0.088 per cent.
Foreign beef . . .	0.16 " "
Veal . . .	0.14 " "
Heart (pig's) . . .	0.0 " "
Heart (bullock's fresh) . . .	0.0 " "

In this set of experiments the meat infusion was fermented with yeast, and in no instance was it possible to obtain a colour with alkaline picrate.

## THE EFFECT OF DIFFERENT SAMPLES OF MEAT UPON THE REACTION CURVE OF A MEDIUM.

These experiments were carried out to determine the difference in effect of freshly killed English meat and foreign chilled meat. Several sets of experiments were carried out, but only one is reported here.

The experiments were made in test-tubes. Two per cent. of peptone and 0.5 per cent. of sodium chloride were added to the meat infusion, made by heating meat with twice its weight of water to 80° C., and filtering.

After adjusting to particular reactions and sterilising, the media were checked with the hydrogen electrode. As will be seen the differences in absolute reaction were very great, due partly, it would appear, to the quality of the meat and partly to the quality of the peptone. All tubes were inoculated with a rapidly growing culture of *B. diphtheriæ*, and a tube taken on each of the specific days for a hydrogen ion concentration determination.

One sees in the present experiment the profound effect obtained by starting the fermentation with an initial hydrogen ion concentration in the neighbourhood of neutrality. Where the initial concentration was pH 7.1 the final reaction became more acid than pH 5.6. pH 7.5 would seem to be the limiting concentration for reversal, and statements of other workers appear to confirm this.

The results are given in Table I.

TABLE I.

Days.	Foreign beef and Parke Davis' peptone.	English beef and Parke Davis' peptone.	Foreign beef and Morson's peptone.	English beef and Morson's peptone.	Foreign beef and Witte's peptone.	English beef and Witte's peptone.
0 .	7.6 .	7.6 .	7.7 .	7.5 .	7.3 .	7.1 .
4 .	7.0 .	6.6 .	6.9 .	7.1 .	6.6 .	7.1 .
7 .	6.9 .	6.5 .	6.9 .	6.6 .	6.1 .	5.6 .
11 .	6.9 .	6.5 .	6.9 .	6.9 .	6.2 .	5.6 .
14 .	6.9 .	6.8 .	6.8 .	6.9 .	6.2 .	5.6 .
18 .	+8.0 .	7.6 .	+8.0 .	8.2 .	6.9 .	-5.6 .
25 .	+8.0 .	7.2 .	+8.0 .	+8.0 .	6.7 .	-5.6 .

The results as given in the above table do not lead to perfectly straightforward conclusions. The initial reaction is obviously not an all-important factor, for while the more alkaline media do reverse, one at least, made with English beef and Parke Davis' peptone, had only reached a pH of 7.6 on the eighteenth day. On the other hand, a medium made with English beef and Morson's peptone, which only had an initial alkalinity of pH 7.5, reversed as quickly and as far as the companion medium made with foreign meat, which started with an alkalinity of pH 7.7. These results confirm what Hartley has found in his study of peptones, viz. that each peptone has individual qualities which must be taken into consideration if the best possible use is to be made of them.

That the quality of the meat infusion may have a very serious effect on the course of a bacterial growth is shown from another experiment in which Parke Davis' and Morson's peptones were combined with two infusions and the



cultures of *B. diphtheriae* allowed to incubate for two weeks. The results were as follows :

	Initial pH.	Final pH.
Parke Davis and foreign meat . . .	6.85	7.82
Parke Davis and English meat . . .	6.79	6.55
Morson and foreign meat . . .	6.89	8.39
Morson and English meat . . .	6.74	6.74

Here one sees that there was no reversal when the English meat infusion was employed, while the results were quite satisfactory with the chilled samples.

#### CONCLUSION.

Chilled foreign meat contains more glucose than fresh English meat, but nevertheless the reversal which occurs in the successful production of toxin takes place more constantly when chilled beef is used.

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## ON VITAMIN UNDERFEEDING.

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THE relations of vitamins to dietetics present two different aspects, one of which has so far been completely neglected. Up to now only the two extreme conditions have received consideration: the effects of a diet from which one or more vitamins are completely or almost completely excluded, and which eventually leads to disease, is contrasted with what is called the "normal" condition resulting from a diet rich in vitamins. These studies have been of great importance in identifying the presence of vitamins in the various articles of food. They have, however, led to a misconception of their mode of action, and of the ætiology of the so-called deficiency diseases. And they have failed to recognise that there exists a condition of "vitamin underfeeding"—as distinct from vitamin deficiency—where the supply of vitamins, though adequate to prevent obvious ill-health and to enable the animal to grow and breed, does not permit the organism to reach its full development. As is to be expected, pregnancy, lactation and infancy are the periods in which the ill-effects of vitamin underfeeding are most likely to make themselves felt, and these periods will be particularly dealt with in this paper. But before considering the importance of this condition it is necessary to subject the

prevalent vitamin conception to a brief criticism. It may be added at once that this criticism in no way detracts from the importance of the vitamins in dietetics, but on the contrary emphasises it.

#### A CRITICISM OF THE PREVALENT VITAMIN CONCEPTION.

The prevalent conception of vitamins is based on the fundamental assumption that a supply of vitamins is necessary for the life of every cell. In what way they act is admittedly not understood. It is clear that they are not a source of energy. It is stated that very minute amounts are sufficient to ensure a normal cell metabolism, and they are not supposed to have any positive action, so that their existence can be recognised only through the effect produced by their absence over prolonged periods. If their supply is withheld diseases develop which are held to be specific for vitamin deficiency. These eventually kill the animal, but according to the orthodox vitamin conception they do so in the absence of pathological lesions sufficiently severe to account for the death of the animal. Death is supposed to be the result of a failure in the vital activities of the general mass of cells in the body.

This view introduces a new pathological conception of the ætiology of disease, and those diseases in which vitamin deficiency is held to be the only ætiological factor have therefore been called "deficiency diseases." Conversely, the occurrence of such diseases in man or animals is taken as indicating a vitamin deficiency as the only ætiological factor. And the absence of these specific diseases in man and animals living on ordinary unrestricted and freely chosen dietaries has led to the comfortable belief that any freely chosen diet is adequate from the point of view of vitamin supply, and that "we need not bother about vitamins."

This conception does not necessarily follow from the facts observed and involves a number of assumptions. The whole evidence of the existence of vitamins is based on the fact that a highly organised animal (a mammal or a bird) dies when vitamins are withheld from the diet. As we pointed out in previous papers (Cramer, Drew and Mottram, 1921, 1922), this observation shows that the organism as a whole requires a supply of vitamins, but it does not necessarily follow from it that all the cells of such an animal require vitamins. And since the adrenal gland hypertrophies in vitamin deficiency, further subsidiary assumptions would be necessary to avoid the conclusion that the cells of the adrenal gland do not require a supply of vitamins, but can maintain themselves and actually grow in their absence. Further observations on the growth of mammalian tissue *in vitro* do not agree with the contention that all the cells of a mammal require vitamins. Drew (1922) has been able to grow such cells in a saline medium for an indefinite length of time if a watery extract of embryo was added to the saline medium. Adult tissues were found to contain much less of the substance or substances capable of maintaining growth *in vitro*. Feeding experiments have failed to show that embryos are particularly rich in vitamins. Moreover, the active substance present in the embryo extract differs from vitamins in being very thermolabile; it is inactivated by heating the extract for 10 minutes to 60° centigrade. Lastly embryo extract cannot be replaced by a yeast extract, which has no effect at all in promoting growth of isolated cells *in vitro*.



When we published our first criticism of the current vitamin conception it was still firmly believed that all forms of cell life, even the lowest, such as bacteria, yeast cells and plant cells, required a supply of vitamins. This view was so firmly held that methods had actually been devised to test for the presence of vitamin B, and estimate it quantitatively, by its effect on the growth of yeast cells. We know now that yeast cells can synthesise vitamin B, and that plant cells can synthesise vitamin A. These recent observations would therefore necessitate a modification of the original contention that all cells require vitamins. Quite recently the curative effect of light in rickets has led to the further assumption that even in a highly organised animal some cells can synthesise vitamin A under the influence of light and pass it into the circulation for the benefit of other cells. It does not seem to have been noticed that this new assumption involves a reversal of the original contention. It may be added that the available experimental evidence fails to confirm this assumption. So far no convincing demonstration has ever been given of a failure in the vital activities of the general mass of cells in animals suffering from a vitamin deficiency. In our observations we have equally failed to get an indication of such a general wide-spread failure. Histologically the finer cytoplasmic structure of the cells remains preserved even in the last stages of vitamin deficiency and no definite nuclear changes could be detected. Physiologically it might be expected that a general failure would make itself felt in the sensitiveness of the sympathetic nervous system. But in experiments (unpublished) carried out with Dr. Samson Wright, it was found that intestinal strips from rats in the very last stage of vitamin deficiency responded as actively to adrenalin as intestinal strips from normal rats. In fact the former were more sensitive than the latter. In view of all this evidence it seems advisable to go back to the experimental facts which established the existence of vitamins, and to realise that these prove only that vitamins are necessary for the life of a highly organised animal as a whole. There is no evidence that the ill-health occasioned by their absence is due to a general wide-spread failure of the vital activities of every individual cell.

In searching for an explanation of this ill-health (Cramer, Drew and Mottram, 1922), we have found, on the contrary, that there are definite lesions in certain tissues, which are specific for each vitamin and which produce specific effects in the organism. There is atrophy of lymphoid tissue and, as a result, marasmus in vitamin B deficiency; there is a thrombopenia and, with it, increased susceptibility to infection in vitamin A deficiency. These same lesions can be produced by agencies other than vitamin deficiency, such as the radiations of X-rays or radium, and when these lesions are thus produced, there is the same effect on the organism as a whole. In other words, the so-called "deficiency diseases" are, like any other disease, the result of specific pathological lesions which can be produced by a variety of agencies of which the deficiency in vitamins is only one.

It follows from this that the production of a disease by withholding a vitamin does not exclude the possibility that the same disease might not be produced by other agencies even in the presence of this vitamin. In the light of this conception the controversy concerning the ætiology of certain so-called

deficiency diseases assumes a new aspect, and the discrepancies between clinical experience and experimental observations disappear.

Lastly, there is the tenet of the current vitamin theory that these substances have no positive action. Our observations (Mottram, Cramer and Drew, 1922) have shown that vitamins produce an immediate stimulating effect on the processes of digestion and assimilation, and that this effect takes place even in a normal organism which has not previously been subjected to any vitamin deficiency. These observations agree with the findings of other observers (Voegtlin and Myers, 1919, Anrep and Drummond, 1921), who showed that vitamin preparations stimulate the activity of the digestive glands and are capable of producing a flow of secretion from them. The important conclusion follows that the various articles of food contain substances capable of stimulating digestion and assimilation, and they contain these substances, which might be called "food hormones," in varying quantities. An analogy may be drawn between the atrophic changes in specific tissues such as the atrophy of lymphoid tissue when the constant stimulus of the food hormone, water-soluble B, is withheld over prolonged periods, and the atrophic changes which take place, for instance, in the uterus after removal of the ovary, and which are generally attributed to the absence of an ovarian hormone. That the complete or almost complete absence of these "food hormones" leads to disease reveals only the negative aspect of their significance. There is also the no less important positive aspect that their presence has a stimulating action on specific functions, and that the different dietaries will differ in the positive stimulating action according to the supply of vitamins they contain, and that the aim must be to ensure a maximum supply.

#### THE EFFECT OF VITAMIN UNDERFEEDING ON YOUNG RATS.

The significance of this conclusion is exemplified in a most striking manner by the difference between a stock of rats kept on our ordinary laboratory diet and a stock of rats receiving the same diet with an ample supply of vitamins. In this laboratory large numbers of rats have been kept for years on a regular diet of bread and water supplemented with boiled maize and rice. On this natural diet they breed freely, they grow, they do not suffer from epidemics, so that the laboratory stock has replenished itself for years with the occasional introduction of fresh stock. Occasionally a litter would fail to grow and sometimes a case of spontaneous rickets has been observed, but such cases are exceptions. On the whole these animals were to all appearances normal healthy animals, which compared favourably with many of the rats obtained from breeders. I had noticed, however, that though these animals grow, their rate of growth is much slower than that given by Donaldson as a standard in his book on the rat. But as soon as material rich in vitamin, such as marmite and cod liver oil, was added to the laboratory diet the animals grew much more rapidly, although even then they did not always attain the standard rate given by Donaldson. The differences in the size of rats of equal age kept on the laboratory diet alone and on the same diet supplemented with vitamin-rich material are remarkable. Details of such observations have been given in a previous paper. These data show that a rat kept on a vitamin-rich diet may



have double the weight of an animal kept on the laboratory diet alone and have a corresponding increase in length. The functional activity of the lymphoid tissue in the former case is considerably increased and there are other differences which need not be detailed here.

Our laboratory diet of bread, rice and maize has, therefore, for years enabled our rat population to lead a normal vegetative and reproductive existence. This diet, which is not very rich in vitamins, contained nevertheless sufficient vitamins for that purpose, but not sufficient to ensure the full development of which the rat is capable. It is more than probable that large classes of civilised mankind live on diets of a correspondingly restricted vitamin content, adequate to prevent obvious ill-health but not adequate to ensure the maximum development. It may be added that our laboratory diet is also poor in calcium. This dietetic fault is, of course, not corrected by the addition of vitamins.

The fact that intestinal digestion is subject to the influence of "food hormones" is a fact of considerable importance which is not yet sufficiently recognised. It is likely to be of particular importance in such a condition as pregnancy where gastro-intestinal disturbances are so easily elicited. It is equally important for the nutrition of the infant. Clinical experience has already shown that in certain types of marasmus in infants, where in spite of careful feeding and treatment the child fails to put on weight, the administration of vitamins in the shape of cod liver oil, malt and orange-juice is capable of rapidly relieving the condition.

#### VITAMIN UNDERFEEDING IN PREGNANCY AND LACTATION.

A study of the work on the metabolism of pregnancy—a detailed account of which will be found in a chapter by Lochhead and Cramer in the forthcoming new edition of the 'Physiology of Reproduction,' edited by Dr. F. H. A. Marshall—reveals as the outstanding change in the pregnant organism that it acquires the power to retain and store nitrogen over and above the needs of the foetus. This process is probably a preparation for the purpose of laying down a store for the period of lactation. Now the fact that an animal increases in size and weight the more rapidly vitamins are supplied to it means in terms of metabolism that vitamins favour the retention of nitrogen. It is therefore to be expected that in pregnancy the characteristic retention of nitrogen should be dependent also on the supply of vitamins. The vitamin factor was unknown when the experimental observations on the nitrogen metabolism in pregnancy were carried out and had therefore not received any consideration. But the data given in the various papers are sufficient to show that the characteristic nitrogen retention of pregnancy is indeed dependent on the vitamin content of the diet. Thus the earliest observations on dogs (by Hagemann and by Jägersoos) showed a loss of nitrogen during pregnancy on a high nitrogen intake, while with a lower nitrogen intake Bar obtained a retention of nitrogen. The diet in the earlier experiments was either lean meat alone, or supplemented by cane sugar—a diet very poor in vitamins. Bar gave meat together with bread and animal fat—a diet richer though by no means abundant in vitamins. The pregnant woman observed by

Hoffström, who showed a remarkably high nitrogen retention, lived on a very varied and vitamin-rich diet, in which milk and butter, for instance, figure largely.

In order to test this important point directly, fresh observations have been carried out on a number of female rats. These rats were kept together with male rats on the laboratory diet of bread and water, maize and rice. In some cases this diet was supplemented by marmite, as a source of the water-soluble vitamin, and cod liver oil, as a source of the fat-soluble vitamin. The weight was taken once a week and as soon as the animal had littered. The litter was weighed separately. The period of gestation was taken from our observations as 23 days and the day of conception calculated accordingly. In this way it was possible to determine whether the mother had lost or gained during pregnancy. In some cases the same animal was observed first on the one diet, then on the other.

The results are given in the following table:

*I. Laboratory Diet + Vitamins A and B.*

No. of rat.	No. of successive pregnancy.	Wt. of mother in grms.		Loss or gain of mother in grms.			Litter.			Interval in weeks between end of lactation and succeeding conception.
		At conception.	After littering.	During pregnancy.	During lactation.	Total.	Total wt. in grms.	No. of young.	Average wt. of single young.	
223	3rd	215	220	+ 5	$\pm 0$	+ 5	45	7	6.5	1
—	4th	225	250	+ 25	- 20	+ 5	45	8	5.6	3
221	2nd	140	165	+ 25	Not determined.	—	33	5	6.6	1
222	—	170	230	+ 60	—	—	45	8	5.6	Not known.
265	1st	160	185	+ 25	+ 10	+ 15	24	4	6.0	Do.
—	2nd	205	230	+ 25	- 25	$\pm 0$	34	6	5.7	12

*II. Laboratory Diet alone.*

—	1st	210	220	+ 10	- 25	- 15	50	9	5.5	Not known.
223	2nd	215	230	+ 15	- 45	- 30	40	8	5.0	4
—	6th	220	230	+ 10	- 40	- 30	48	6	8.0	3
221	1st	145	145	$\pm 0$	- 25	- 25	60	9	6.7	Not known.
266	—	220	220	$\pm 0$	Not determined.	—	Litter partly eaten.			Do.

It should be noted that the observations were made on rats which had not yet attained their full weight. The table shows that on the vitamin-rich diet the rats complete pregnancy as a rule with a considerable gain in weight. On the laboratory diet alone the gain in weight is absent or much less. The difference becomes more marked in the period of lactation. Lactation imposes an even heavier drain on the mother, so far as energy requirements are concerned, than pregnancy. For, as Murlin has shown, the energy requirements of the newborn are much higher than those of the embryo immediately before birth. This is due to the fact that the newborn requires additional



energy to maintain its body temperature against a cold environment. We find accordingly a considerable loss of weight on both diets. But this loss is slight on the laboratory diet enriched with vitamins, while it is considerable on the laboratory diet alone. Taking periods of pregnancy and lactation together, one finds that the weight of the mother remains practically unaltered when she receives the laboratory diet enriched with vitamins, but suffers a considerable loss of weight on the laboratory diet alone. This may be of serious import when pregnancies rapidly succeed each other, so that a new pregnancy begins before the period of lactation of the preceding pregnancy is completed. In this series of observations care has been taken to let at least one week elapse between the end of the period of lactation and the beginning of the next pregnancy.

#### VITAMIN-UNDERFEEDING IN INFANTS.

The most important aspect of this problem refers, not to the mother, but to the offspring. The vitamin content of the milk is dependent on the vitamin supply to the mother. There is a difference between two litters, of which one is suckled by a mother which receives the laboratory diet alone, while the other is suckled by a mother kept on the laboratory diet enriched with vitamins. This difference becomes greater if the dietetic differences are maintained through successive pregnancies. This difference manifests itself not only in the rate of growth, but also in the general development, such as, for instance, the growth of the fur. And this difference persists and becomes more marked when after weaning the two litters the difference in the two diets is maintained. There is then a very distinct difference in the rate of growth. This is the most obvious effect, but the effect in growth is only a crude manifestation. More essential and more important differences exist between two such litters, although there is nothing apart from the difference in weight and size to indicate those differences, either macroscopically or microscopically. Young rats kept on the laboratory diet alone look perfectly healthy and show no obvious signs of disease. But a significant difference reveals itself when rats from two such litters are now deprived of the fat-soluble vitamin. Animals derived from a litter kept on the laboratory diet alone will at once stop growing, and within 8-10 weeks at the latest develop the typical eye lesion and look ill and emaciated. But if the animals have since birth been kept on the laboratory diet enriched with vitamins they may continue to grow for a considerable time. Their growth in the absence of the fat-soluble vitamin may even be more rapid than that of normal rats receiving the laboratory diet. They look perfectly well and healthy for three or four months, and only develop the typical eye lesion two or three months later than the rats from the other litter. Two of these rats were kept on the completely vitamin A free diet for nine months before the eye lesion developed, and the only obvious evidence of the absence of the fat-soluble vitamin was that they did not attain their full weight, and in that eventually they did not look quite so sleek as a normal animal. Fig. 1 represents such an experiment. Three rats from a litter ("Y"), kept on the laboratory diet alone, and three rats of the same age from another litter ("Z"), kept on the laboratory diet plus vitamins, were placed together in one cage. They were fed on the vitamin-free basal ration, to which

the water-soluble vitamin was added in the form of marmite. The six rats lived therefore under exactly the same conditions. The rats from litter "Y" ceased growing immediately. Two developed keratomalacia within 8-9 weeks and died; the third died of pneumonia in the ninth week. The rats from litter "Z" continued to grow actively, looked well, and did not develop the eye lesion within the twenty weeks during which the experiment was continued, although they were living in the same cage as the rats with the eye infection and were therefore exposed to the same extraneous risks of the infection. The animals which show the least resistance to the infection are those which had been subjected to vitamin underfeeding since birth. It might be argued that this difference is simply due to a storage of the fat-soluble vitamin A in the tissues of the animal during infancy when they receive a vitamin-rich diet. But such an explanation does not account for the fact that such animals continue to grow as quickly when the vitamins are withheld as animals which, after having been subjected to vitamin underfeeding since birth, are placed, when half-grown, on a vitamin-rich diet. In fact, sometimes animals from a "vitamin-rich stock" may grow when placed on a vitamin-free diet even more rapidly than animals from a "vitamin-underfed stock" when placed on a vitamin-rich diet.

Data of another such experiment with similar results have been given in a recent paper (Cramer, Drew and Mottram, 1922). In that paper it was shown that the effect of withholding the fat-soluble vitamin A is to reduce the number of blood-platelets by 60 to 80 per cent. of the normal. Associated with this thrombopenia there is a diminution in the resistance of the animal to certain types of bacterial infections. Rats which have received an abundant supply of vitamins since birth do not undergo this reduction in the number of platelets as readily and as quickly as animals from a "vitamin-underfed stock," and also retain their resistance to those infections over a longer period.

These observations should not be taken as implying a criticism of the methods of the so-called quantitative estimation of vitamin A, although they probably account for many of the discordant statements which have been made concerning the content of certain foodstuffs in this vitamin. Our curves show that though the degree of sensitiveness to a deficiency in this vitamin varies widely in normal animals taken at random, a very constant degree of sensitiveness can be obtained if the dietetic conditions to which the animal

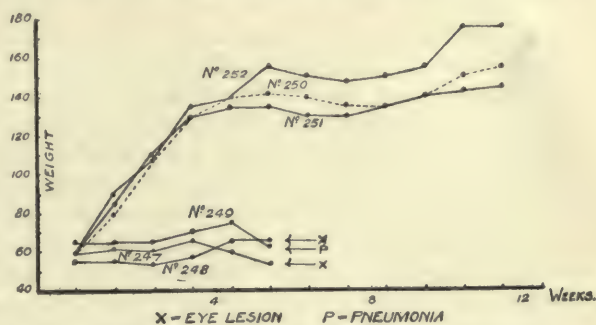


FIG. 1.—Weight curves of six rats from two litters kept in the same cage on a diet free from vitamin A (basal ration and salt mixture and marmite). Rats No. 247, 248, 249 belonged to one litter taken from a vitamin-underfed stock and had themselves been kept since birth on the routine laboratory diet. Rats 250, 251 and 252 belonged to another litter taken from a stock kept on the routine laboratory diet plus marmite and cod liver oil and had themselves received this diet since birth. The curves illustrate the delayed effect of vitamin under-feeding in early life.



has been subjected since birth have been kept constant, and can be varied at will by varying those preliminary dietetic conditions. The point we wish to emphasise is that the negative conception of the mode of action of vitamins should be replaced by the positive conception that these substances have specific positive, stimulating actions, and that the varying degree in which an organism has been subjected to the action of these food hormones, especially during infancy, produces lasting differences in certain tissues. These differences do not necessarily manifest themselves in obvious ill-health: they exist in a latent form between apparently normal animals, using the word "normal" in the sense in which it is usually used as indicating the absence of obvious disease.

#### SUMMARY.

Observations have been made on a stock of rats which have been kept through many generations on a natural diet, the vitamin content of which, though restricted, is adequate to enable them to grow and breed and to prevent the occurrence of obvious ill-health. A comparison of rats from these stocks with animals from a stock fed on the same diet supplemented with an abundant supply of vitamins shows that there is such a condition as "vitamin underfeeding" and that it may occur on a natural diet. Such vitamin underfeeding does not lead to any obvious ill-health: the animals are in appearance normal, healthy animals. But vitamin underfeeding, especially if it has occurred in infancy, impresses itself upon the organism as a lasting weakness which only manifests itself when the organism is exposed to a strain. Hence the importance of insuring an abundant supply of vitamins in the food, especially to the pregnant and lactating mother and to the growing child.

The fact that vitamins have a positive, stimulating drug-like action and thus act as food hormones is put forward to replace the prevalent conception of their mode of action.

These conclusions open up a social aspect of the vitamin problem which has hitherto not been recognised, but which is at least as important as the actual production of diseases by a severe vitamin deficiency. They suggest that the physical make-up of a community is determined largely by the ease and regularity with which an abundant supply of vitamins is secured to the pregnant and nursing mother and to the growing child.

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## THE LABORATORY PREPARATION OF A PURIFIED HÆMATOXYLIN.

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It is well known that many of the commercial preparations of hæmatoxylin have proved unsatisfactory as histological stains. Indeed, since the war, there has been no good hæmatoxylin on the market. It was found that the most unsatisfactory feature of all the commercial samples was a diffuse pink staining over the entire specimen, which interfered with the black staining of the hæmatoxylin when used by Heidenhain's method. In addition to the admixture with the pink compound, it was found that the hæmatoxylin were too fully oxidised, which apparently interferes with their staining properties. The ideal preparation seems to be a solution containing the hæmatoxylin as a leuco-base which is oxidised in the tissues themselves.

Hæmatoxylin may be reduced in alcoholic solution by means of finely divided zinc dust. The commercial hæmatoxylin is dissolved in absolute alcohol to form a 5 per cent. solution, five times the weight of zinc dust is added to the solution, and the whole boiled gently for two hours in a flask fitted with a reflux condenser. Not all specimens of zinc were found suitable, as much of that on the market is far too coarse, and is in fact zinc filings and not dust. The zinc should be in as finely divided a state as possible. During the boiling the hæmatoxylin loses its colour, the pink staining substance is thrown out of solution, and is adsorbed by the zinc. After boiling for two hours the hot solution is rapidly filtered through paper into a clean dry bottle. This filtration is best carried out either in an atmosphere of hydrogen or of coal gas. The solution should come through the filter either colourless or with only a faint yellow tint. It is convenient so to arrange the amount of hæmatoxylin that the final filtered solution is of about 5 per cent. strength, as this can be readily diluted to form the ordinary laboratory stock solutions. Solutions made up from this reduced hæmatoxylin are nearly colourless at first, but stain perfectly well, iron-hæmatoxylin preparations showing clean jet black pictures, with no diffuse staining whatever. Ehrlich's acid hæmatoxylin made up in the usual way with this purified stain is very good. For Weigert's iron hæmatoxylin dilute the acid ferric chloride solution with an equal volume or more of 0.5 per cent. hydrochloric acid in water. Five volumes of this diluted iron solution is added to five volumes of 0.5 per cent. hæmatoxylin in 95 per cent. alcohol.

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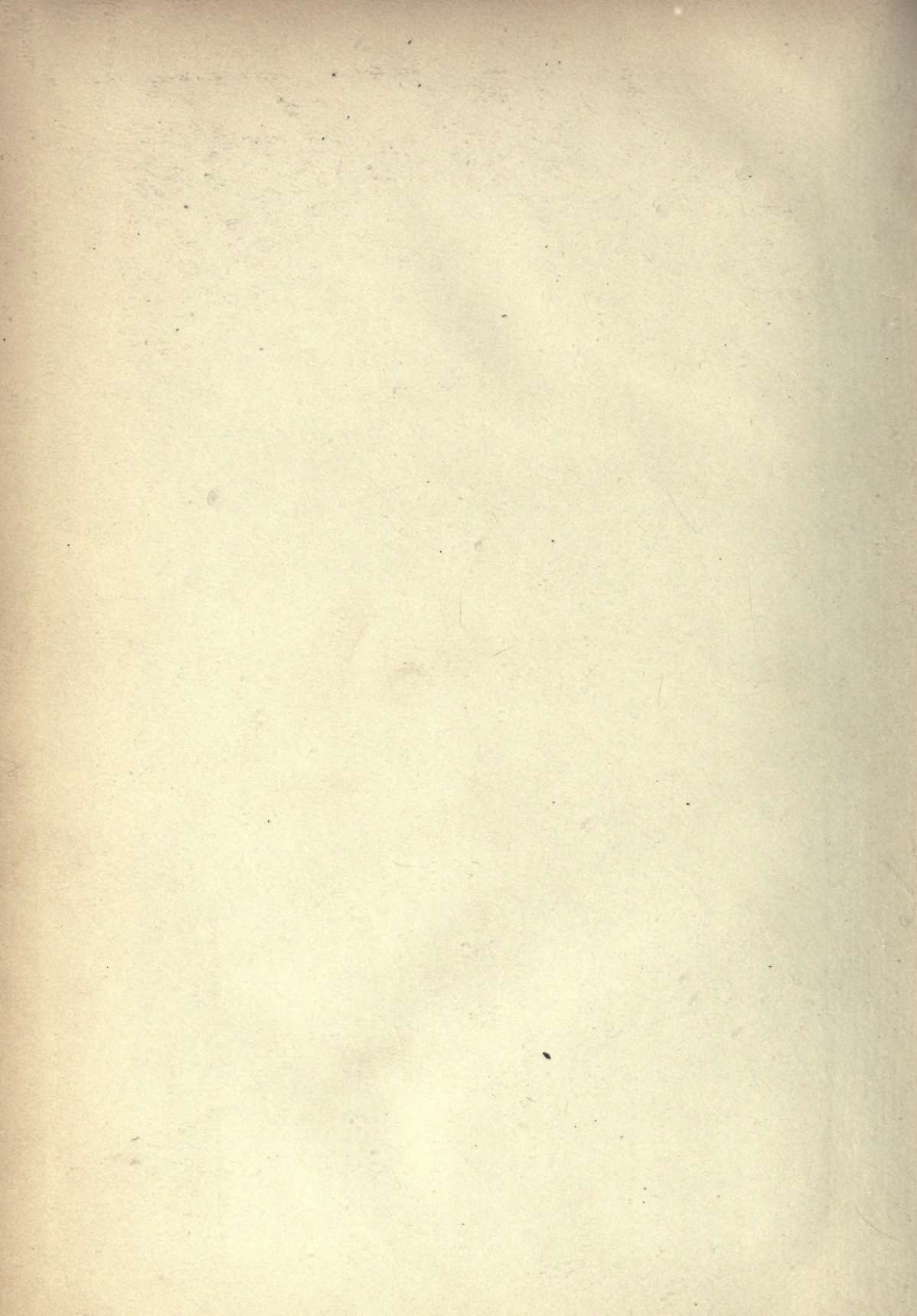














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